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14. ABSTRACT

In this project, we have demonstrated multi-component molecular targeting of surface receptors (IGF1R and Her2) and NIR dosing of cancer cells using SWCNT. While previous studies have shown the transport of DNA into cells using nanotubes, in this study we show multi-component molecular targeting of both IGF1R and Her2 surface markers in cancer cells using single wall carbon nanotubes. IGF1 and Her2 specific antibodies conjugated to the SWCNT were used to target their corresponding receptors in cells and internalize the SWCNT. The cells were then dosed with NIR 808 nm photons at ~800 mW/cm2 for 3 minutes. Cells that were treated with non-specific antibody-SWCNT hybrids survived the NIR dosing compared to the dead cells with anti-IGF1- anti-Her2-SWCNT hybrids. The amount of energy consumed for cell killing was estimated to be as small as ~200 nW per cell, which is an order of magnitude smaller than competing techniques. Quantitative estimates showed that cells incubated with SWCNT-anti-Her-anti-IGF1R antibodies were completely destroyed while 80% of the non-specific-antibody-SWCNT hybrids treated cells were still alive. These results indicate that SWCNT could be used as biological transport agents and the high optical absorbance in the NIR are capable of killing cancer cells with minimum collateral damage. Further, western blots indicated the relevance of targeting IGF1R in MCF7 cancer cells and Her2 in BT474 breast cancer cells. This study is another clear example of molecular targeting and cell killing approach that can improve therapeutic efficacies. These tools can also be applied to traditional surgical oncology to improve the probability of killing cancer cells whose microscopic foci are often left behind in surgery.

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Integrated Molecular Imaging and Therapy for Breast Cancer

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I. Introduction

In 2008, invasive breast cancer will attack approximately 190,000 US women, and this malignancy will take the lives of approximately 41,000 patients. Breast cancer is the leading cause of cancer in US women (excluding skin cancers). Diagnostic systems that can detect cancer at an early stage can be a priceless gift to patients suffering from this disease. With progress in the area of nanotechnology, it is now possible to synthesize nanotubes and nanowires approaching the size of proteins or DNA or other functional biological molecules (1.5 nm to 10 nm). The discoveries of quantum phenomena and surface plasmon resonance in nanomaterials have produced far more important results in imaging and therapeutics and are creating a revolution in the area of biology and medicine. Keeping these advancements in mind, this proposal presents new concept of integrating molecular imaging and therapy in one step that could revolutionize oncology. By using the distance dependent optical color changing property of gold nanoparticles and integrating this principle with materials such as bucky balls and nanotubes that show high optical to thermal transitions in near infra-red region of the electromagnetic spectrum, one can create a composite nanoparticle that can do simultaneous imaging and killing of breast cancer cells. Coating these nanoparticles with anti-oncogene specific antibodies can be a powerful approach towards molecular targeting, molecular imaging and killing of breast cancer cells. BT474 cancer cells overexpress Her2 and MCF7 breast cancer cells overexpress the IGF1 receptor [1-2].

Objectives: The objective of this research is to integrate molecular cancer imaging and therapy in one step that would enable early detection and killing of cancer cells at the molecular level. The hypothesis used here is as follows: Gold nanoparticles have been known to change color from red to blue when they aggregate in colloids. This is due to plasmon-plasmon interactions between locally adjacent nanoparticles and has been observed in the past [3] but never explored for direct cancer imaging. We are using this principle to create a composite nanostructure that uses a bucky ball or nanotube as a core for photo-thermal cell killing and 10-20 nm gold coating for targeted molecular imaging. Molecular imaging is achieved by tagging the gold surface with Her2 and IGF1R specific antibodies and targeting them to their corresponding receptors in cancer cells. When the antibodies dock to their receptors in cancer cells, the cell surface will gradually begin changing color due to the aggregation of nanoparticles and plasmon-plasmon effect [3]. The change in color of the surface of the cell will indicate cancer and the intensity of

the color will indicate the progression of cancer. Once imaged focusing near infra-red (NIR) pulses to the cells will only kill the cells that have changed color due to the strong optical-thermal transitions in nanotubes [4] and bucky balls from the core thereby heating and killing the cells.

II Body

Integrated Molecular Targeting of IGF1R and Her2 Surface Receptors in Breast Cancer Cells and Photodynamic Therapy using Single Wall Carbon Nanotubes

Despite outstanding progress in the area of cancer biology, significant challenges remain in administering highly selective, targeted anti-cancer therapy. It is striking to note that only 1 to 10 parts per 100,000 intravenously administered monoclonal antibodies reach their parenchymal targets *in vivo* [5]. Nanoparticles [6-7], nanoshells [8-9] and nanotubes [10-11] have been shown in the past to be quite applicable for cancer imaging and therapy. Subcellular nanostructures exhibiting advanced physical properties for biological applications can have an important impact on the introduction and delivery of DNA, proteins, and drug molecules into the living cells [12]. In addition, the unique electronic [12-13], optical [14-16], thermal properties [17], and the subcellular size of nanostructures makes them very attractive for multi-component targeting of surface receptors to enhance the efficacy of anti-cancer agents.

Nanoscale materials with opto-thermal transduction principles have been shown to be an interesting approach for cancer cell destruction. Nanotubes are highly suitable for this technique because of their high efficiency in optical absorbance and photo-thermal cell destruction [12]. The amount of light energy used is quite small compared to competing techniques as the optical absorbance of nanotubes is high in the 700-1100 nm near infrared (NIR) window. This has been recently used to demonstrate many new types of optical sensors and actuators based on the photo-mechanical actuation of carbon nanotubes [18-20]. Therefore multi-component targeting with photo-thermal cell destruction might lead to the development of cancer therapeutics, micro-surgery and cell repair machineries.

While nanoshells have been targeted to the surface receptor of cancer cells in the past [4], in this report we show the integrated targeting of both IGF1R and Her2 surface receptors and photothermal cell destruction using single wall carbon nanotubes. Multi-component targeting of more than one surface receptor can lead to higher efficacies in therapeutics. Past studies involving SWCNT have only targeted folate receptors that are universally expressed on cell surfaces [12]. In contrast, IGF1R and Her2 are both relevant surface markers that are overexpressed in a wide variety of cancer cells compared to normal cells and therefore have great potential for sensing, imaging and therapy of cancer. Circulating cancer cells often express characteristic cell surface markers. Human MCF7 ER+ breast cancer cells exhibit high expression of the insulin-like growth factor 1 receptor (IGF1R), while BT474 ER- cancer cells have lower expression of IGF1R but high expression of human endothelial receptor 2 (Her2) [1].

2. Experimental Details:

2.1 Preparation of SWCNT Solution:

1 mg/mL of SWCNT (Nano-Lab, Lot number FH-P 071706) in phosphate buffered saline solution (PBS) was agitated for one hour under room temperature in ultrasonicator (Fisher Scientific FS60H) to break the SWCNT bundles into individual nanotubes. The solution was then microcentrifuged at 10,000×g for 15 minutes. Sediments containing mostly large

conjugations of SWCNT were discarded and supernatants were collected. To closely examine the dispersion of SWCNT, samples were prepared for transmission electron microscope by dropping 1 μL of such solution on top of a TEM grid. TEM images of the SWCNT were taken from different parts of the solution to ensure that SWCNT were uniformly dispersed within the solution.

2.2 Antibody Functionalization of SWCNT:

5 mg 1-pyrenebutanoyl succinimide was dissolved in 50 mL of methanol with gentle agitation under room temperature. 1 mL solution was mixed with 1 mL SWCNT solution in PBS and kept at 20ºC for 30 minutes to allow the reaction between the reactants. 5 mg PEG (Acros Organic, M.W. 8000) was diluted in the solution to form a self assembly monolayer (SAM) on unoccupied sites so that the SWCNT were insulated from outside liquid to prevent undesirable binding with other bio-molecules. The already functionalized SWCNT were then filtered, collected, triple rinsed, re-suspended in PBS and kept at 4ºC for storage. Such a protocol ensures adhesion of SWCNT and 1-pyrenebutanoyl succinimide, the removal of excess reagents, and allows the complex to be stored for long periods. Non-specific mouse anti human myeloma IgG, anti-IGF1R mouse monoclonal antibody and anti-Her2 (Merck Bioscience, Calbiochem Inc.) were prepared by diluting 1 mg/mL monoclonal antibody solution with PBS. Following this, 1 mL of SWCNT solution was mixed with 1 mL of monoclonal antibody solution (IGF1R or Her2) and the solution was allowed to incubate for one hour at room temperature and microcentrifuged. The sample was triple rinsed to remove excess antibodies in the incubation liquid. These procedures yield two different solutions containing SWCNT-anti-IGF1R antibodies and SWCNT-anti-Her2 antibodies conjugates. Samples for microscopy were then prepared by dropping a small droplet on top of the TEM grids and several samples were investigated for antibody functionalization using TEM to ensure the repeatability of the antibody adsorbing effect.

2.3 Toxicity of functionalized single wall carbon nanotubes

Since cancer cell killing techniques may be applicable *in vivo*, it is important to understand the toxicity of nanotubes. Nanotubes were claimed to be toxic by injecting 15 mg/ml into the lungs of a mice, which died within a week after injection due to asphyxiation [22]. Nanotubes belong to the same family as fullerenes and fullerene derivatives have been shown to be non-toxic for medical applications. Recently water soluble nanotubes have shown to be non-toxic and there is data indicating that nanotube derivatives are non-toxic too [23]. By chemically modifying the surface of nanotubes with phenyl, COOH and OH groups nanotubes have shown to be non-toxic and further optical and atomic force microscopy show direct contact between cellular membranes and water-dispersible SWNTs [18]. Our experiments of preparation of SWNT initially with PBS and 1-pyrenebutanoyl succinimide in methanol are expected to have plenty of OH and COOH groups that make it less toxic. The presence of PEG to inhibit non-selective attachment also reduces the toxicity of nanotubes greatly due to the presence of OH groups.

To examine the potential toxicity of SWNT a cell viability assay was performed. MCF7 cells were incubated with functionalized nanotubes using the same procedure mentioned above in 2.2. 5 mg 1-pyrenebutanoyl succinimide was dissolved in 50 mL of methanol with gentle agitation under room temperature. 1 mL solution was mixed with 1 mg/mL SWCNT solution in PBS and kept at 20ºC for 30 minutes to allow the reaction between the reactants. 5 mg PEG (Acros Organic, M.W. 8000) was diluted in the solution to form a self assembly monolayer (SAM) on unoccupied sites so that the SWCNT were insulated from outside liquid to prevent

undesirable binding with other bio-molecules. The functionalized SWCNT were then filtered, collected, triple rinsed, re-suspended in PBS. MCF7 cells were incubated with the functionalized nanotubes, isolated by centrifugation and observed after 48 h. MCF7 cells with out nanotubes acted as control experiments. Following incubation for 48 hours, trypan blue dye was used to investigate cell viability.

2.3 Fluorescent SWCNT-antibody Hybrids:

300U Alexa Fluor 488 phalloidin and 555 (Invitrogen) were dissolved by 1.5 mL methanol and kept at -20ºC for storage. 10 mL SWCNT in PBS at 0.1 mg/mL was mixed with 10 μL Alexa Fluor 488 to deliver green color under confocal laser excitation. Similarly, 40 μL pure antibody solutions at 1 mg/mL were dyed red with 10 μL Alexa Fluor 555. The two mixtures were kept at room temperature for 30 minutes to allow the optimum reaction. The two solutions were mixed following previously mentioned protocol to form Fluor 488-SWCNT-antibody-Fluor 555 complexes for further cell endocytosis.

In order to remove the excessive fluorescent dyes the solution was microcentrifuged at 10,000×g for 5 minutes. Bottom solution containing mostly the heavy Fluor 488-SWCNT-antibody-Fluor 555 conjugates was collected and re-suspended in PBS.

2.4 Cell Culture and Incubation with SWCNT:

Human BT474 and MCF7 breast cancer cells (ATCC, Germantown MD) were incubated in DMEM supplemented with 2 mM glutamine, 5000 U/mL penicillin, 50 μg/mL streptomycin and 10% fetal bovine serum at 37ºC under 5% CO2 for 48 hours before experiments. MCF7 cells were further supplemented with 7.5 nM 17-β-estradiol (Sigma). Cells released by EDTA went on microcentrifuge at 100×g for 5 minutes. Precipitates containing the cells were collected and re-suspended in PBS. 4 μL of SWCNT-anti-IGF1R antibody solution was mixed with 4 μL of cells, incubated for one hour. Following this, 4 μL of this solution was then mixed with the same amount of SWCNT-anti-Her2 antibody solution, incubated for an hour and then micro-centrifuged. This protocol ensured multi-component targeting of both IGF1R and Her2 before photon thermal dosing of cancer cells. SWCNT-anti-IgG non-specific hybrids were obtained through similar procedures.

Two control experiments were investigated to show binding of SWNT to the cell membrane and internalization through receptor specific antibodies. In one set of experiment, nanotubes were terminated with PEG to prevent binding of biomolecules (1-pyrenebutanoyl succinimide was not adsorbed on the side wall of SWNT which enables binding of antibodies). Such conjugate was mixed with IGF1R specific antibodies that were tagged to Alexa Fluor 555. The resulting suspension was then incubated with MCF7 cells for 30 minutes. Another set of experiments, SWNT was conjugated with Alexa Fluor 488 and incubated with MCF7 cells directly for 30 minutes. The resulting suspension was micro-centrifuged, rinsed and imaged using confocal microscopy.

2.5 Photo Thermal Therapy:

Following incubation of SWCNT-antibody complex with the cancer cells, laser light of 808 nm at 800 mW/cm^2 was dosed for 3 minutes to all the samples. After laser excitation, membrane permeability was investigated by adding 0.4% Trypan blue in PBS. Optical microscopy was used to record the images of cell viability, membrane permeability and nanotube binding. The lasers used were tunable from 50 mW/cm^2 to 1 W/cm^2 and intensities were calibrated by power meters. A custom built circuit was used for closed loop control of the lasers. The laser spot size was about 1 cm for the photothermal dosing of samples, which ensured that all the cells in the titer plate received uniform photon dosing.

3. Results:

The SWCNT were about 1.4 nm in diameter and the typical length varied from 500 nm to 1 μm (Figure 1). The shorter SWCNT tend to be straighter with fewer curves which makes endocytosis easier. The functionalization scheme of antibodies is shown in Figure 2. This procedure worked very well for attachment of both specific and non-specific antibodies on the nanotube surface. Transmission electron microscopy (TEM) of the antibody functionalized SWCNT gives us direct confirmation that the antibodies were attached to the SWCNT on many different sites as imaged in Figure 3. This has also been confirmed in the past using atomic force microscopy and confocal microscopy [24]. Potentially each group of carbon atoms equal to the cross-sectional area of the constant region of a mAb constitutes a binding site in the SWCNT that can act as a biological carrier to transport macromolecules inside the cells. Past AFM studies have shown nanotube-antibody hybrids to be 5-8 nm high [24]. Subtracting the diameter of the nanotube ~1.4 nm, this makes the effective height of the antibodies atop nanotube ~3-6 nm. From TEM images presented in Figure 3, it can be observed that each one of the antibody aggregates varies between 66-100 nm. Hence it is expected that about 10-25 antibody molecules per site has been aggregated. Since the nanotubes conjugated with antibodies were rinsed three times before imaging, it is expected that all the antibody aggregates are covalently bonded to the carbon nanotubes. This shows that higher number of antibody molecules per site can be attached to SWNT with out loss of activity for drug delivery.

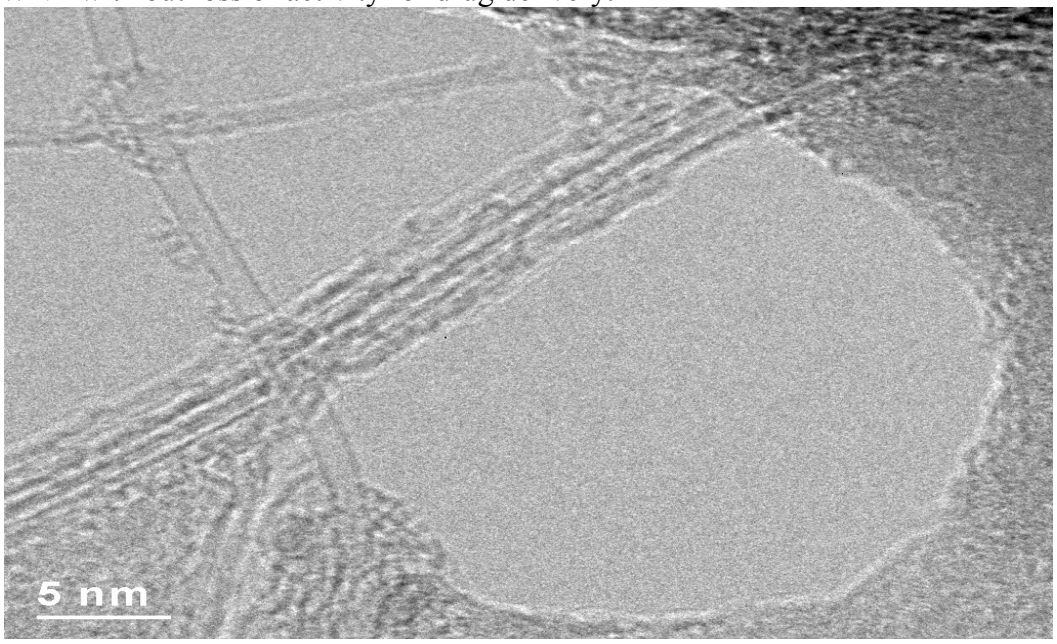

Figure 1: Transmission electron micrograph of single wall carbon nanotube about 1.4 nm in diameter. The single-layer sidewall is clearly visible.

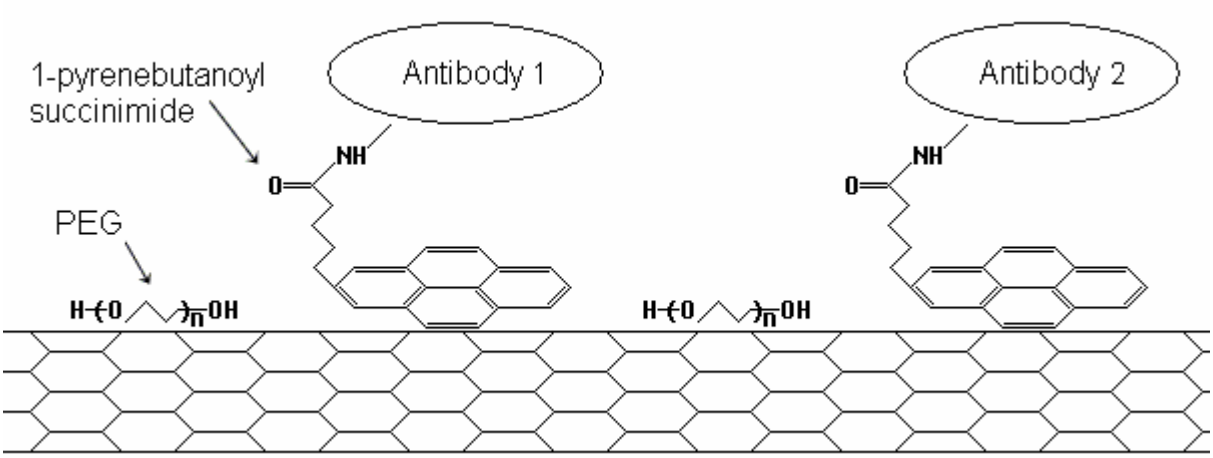

Figure 2: Functionalization schematic of single wall carbon nanotubes with monoclonal antibodies. 1-pyrenebutanoyl succinimide was adsorbed on the nanotube through π-π stacking. The succinimidyl ester can react with available lysine sidechain amines on antibodies to form amide bonds. PEG acts as an insulator to prevent potential bio-fouling.

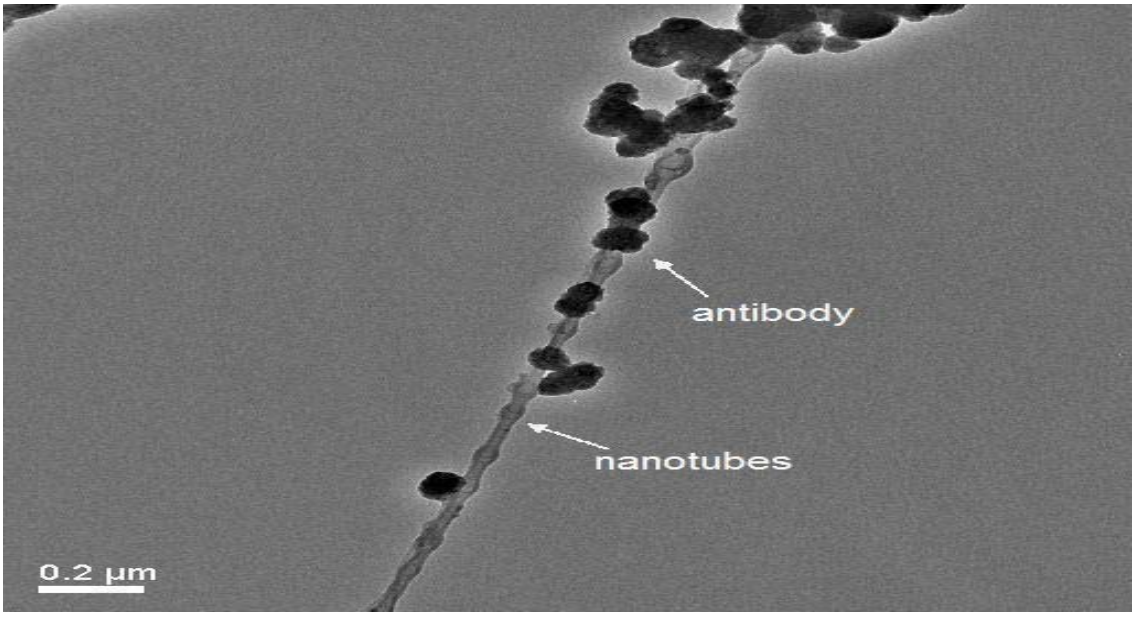

Figure 3: Transmission electron micrograph of the functionalization of monoclonal antibody aggregates specific to IGF1 receptor in breast cancer cells. Each site consists of 10-25 antibody molecules.

One of the most intriguing observations that we made is how the SWCNT get internalized into the cells. Optical and confocal images obtained after the incubation of SWCNT-antibody conjugates with the cells demonstrated the SWCNT were readily internalized into the cells over large areas as shown in Figure 4. The dark areas seen on the optical images were regions of internalized SWCNT. Trypan blue-excluding cancerous cells were also optically imaged as control group.

Cell viability assay was performed and the results are shown in Figure 5. MCF7 cells incubated with single wall carbon nanotubes for 48 hours exhibited little toxicity. The groups containing nanotubes exhibited about 10% cell death and the control group with out nanotubes exhibited about 5% cell death. Most of the cells in both these groups are viable as can be seen by the lack of penetration of Trypan blue dye into the cell membrane. While some cells in the nanotube group show faint blue color, these are on the surface of the cells and not penetration into the cell membrane showing the viability of these cells treated with nanotubes. These results show that functionalized nanotubes themselves exhibit little toxicity to breast cancer cells under study.

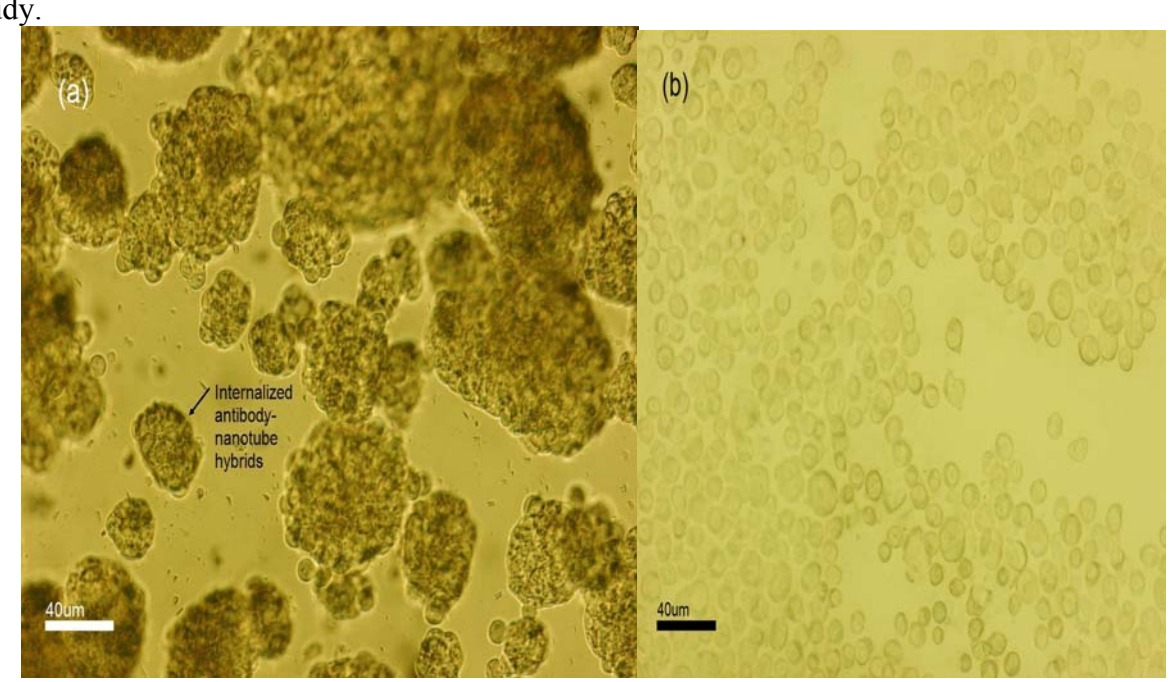

Figure 4: (a) internalization of IGF1R-nanotube hybrid into MCF7 breast cancer cells. The dark regions are the places of internalized nanotube-antibody hybrids. (b) control group of cancer cells without SWCNT internalization.

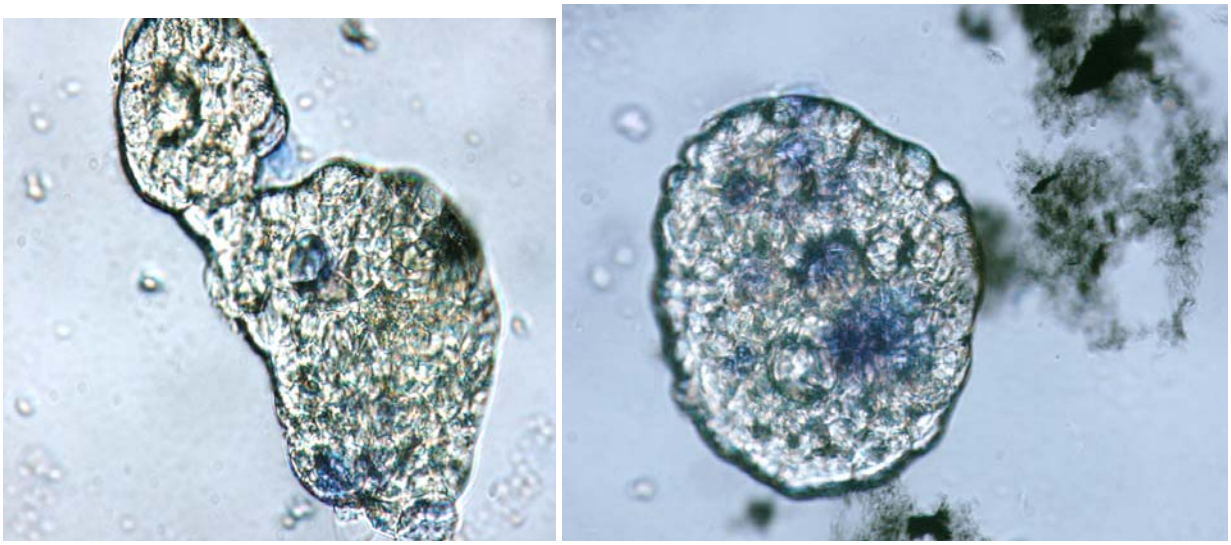

Figure 5 Cell viability assay; (a) control group of MCF7 cancer cells with out functionalized nanotubes, (b) MCF7 cancer cells incubated with functionalized SWNT for 48 hours followed by trypan blue exclusion

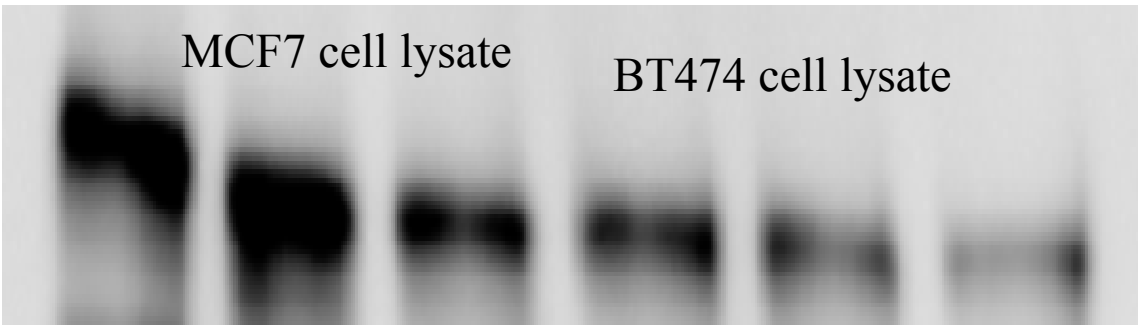

Figure 6: Western blots of IGF1R in BT474 versus MCF7 cells showed band ratio of 0.466 ± 0.241 (n = 6, relative to GAPDH)

It should be noted that human MCF7 ER+ breast cancer cells exhibit high expression of the insulin-like growth factor 1 receptor (IGF1R), while BT474 ER- cancer cells have lower expression of IGF1R but high expression of human endothelial receptor 2 (Her2). While there are several studies that show the over expression of Her2 in BT474 cells [25], western blot analysis was performed for showing the overexpression of IGF1R in MCF7 versus BT474 breast cancer cells. This result is presented in Figure 6. It can be observed that MCF7 breast cancer cells have higher overexpression of IGF1R which is therefore concluded as a relevant surface marker for molecular targeting.

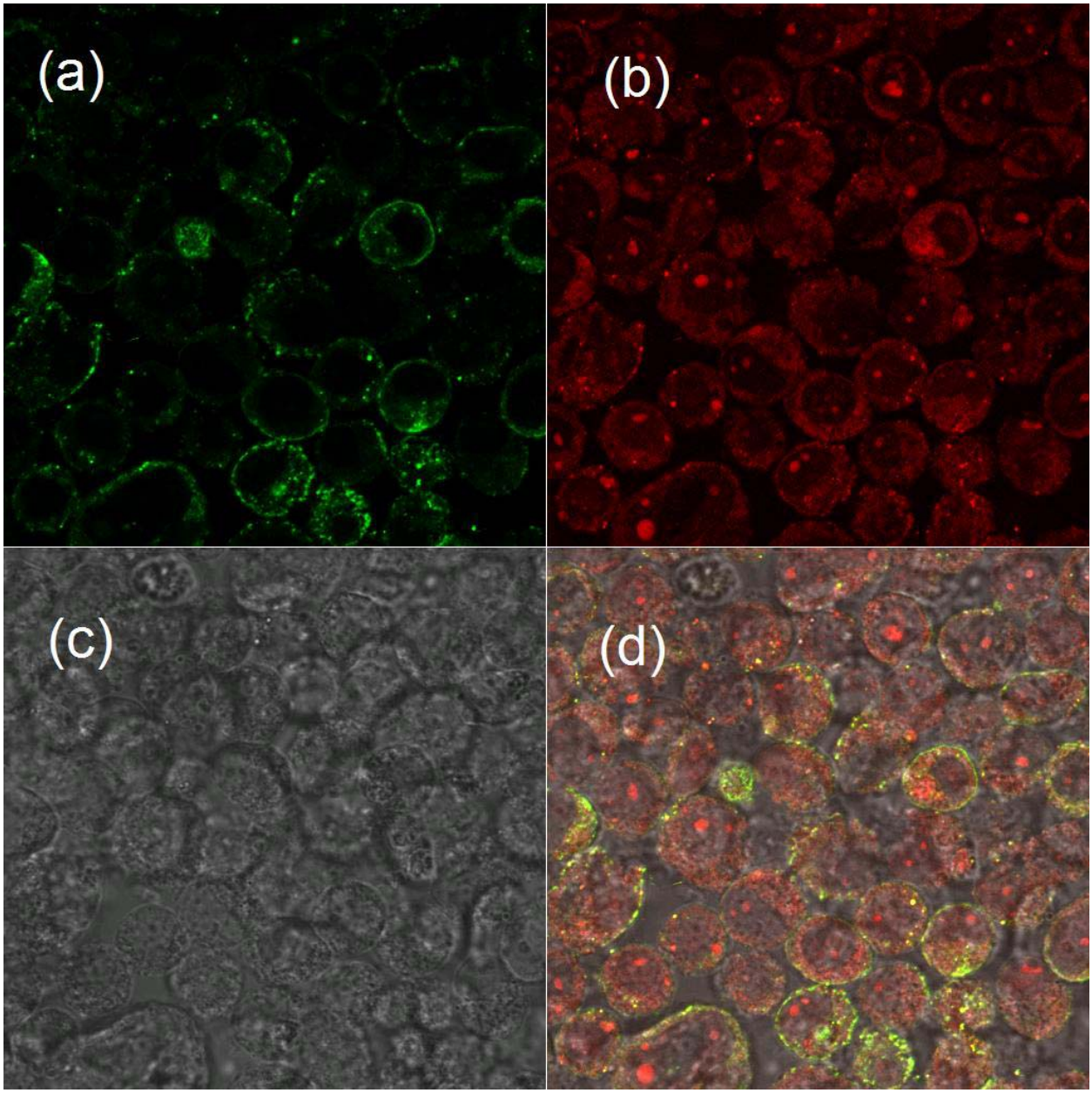

Figure 7. Confocal microscopy of specific antibody facilitated SWCNT internalization of MCF7 cancerous cells. SWCNT and IGF1R antibody were tagged with Alexa Fluor 488 and Alexa Fluor 555 dye respectively. The green (a) and red (b) are fluorescent SWCNT and antibody respectively. By overlapping (a), (b) and optical image (c), the facilitated SWCNT endocytosis could be confirmed by image (d).

Cancer cells incubated with fluorescent specific-antibody-SWCNT complex were imaged by confocal microscopy in Figure 7. Images were obtained at the same spot but using different filters. SWCNT functionalized with Alexa Fluor 488 appeared green inside the cell and on the edge of the cell membrane while Alexa Fluor 555 delivered red color to the antibodies and observed mostly inside the cells. The less intense green fluorescence was caused by the limited binding effect of the dye and SWCNT. Curvature of the SWCNT also made them conjugate on

the cell membrane which was confirmed by the bright green edge of the cells. Staining of cells with internalized SWCNT with Trypan blue did not indicate cell death after incubation. No difference in the internalization was observed for IGF1R and Her2 specific antibodies that were functionalized on the surface of the SWCNT. This process is also continuous and with long periods (>30 minutes) of time, the SWCNT get highly internalized into the cells due to the binding of the antibodies to their corresponding receptors. Non-specific antibodies did not show such internalization but were attached to the circumference of the cell clusters. More studies on the internalization of nanotubes with non-specific and IGF1R and Her2 specific antibodies showed clear internalization of specific antibody nanotube complexes into the cells. This is presented in Figure 8.

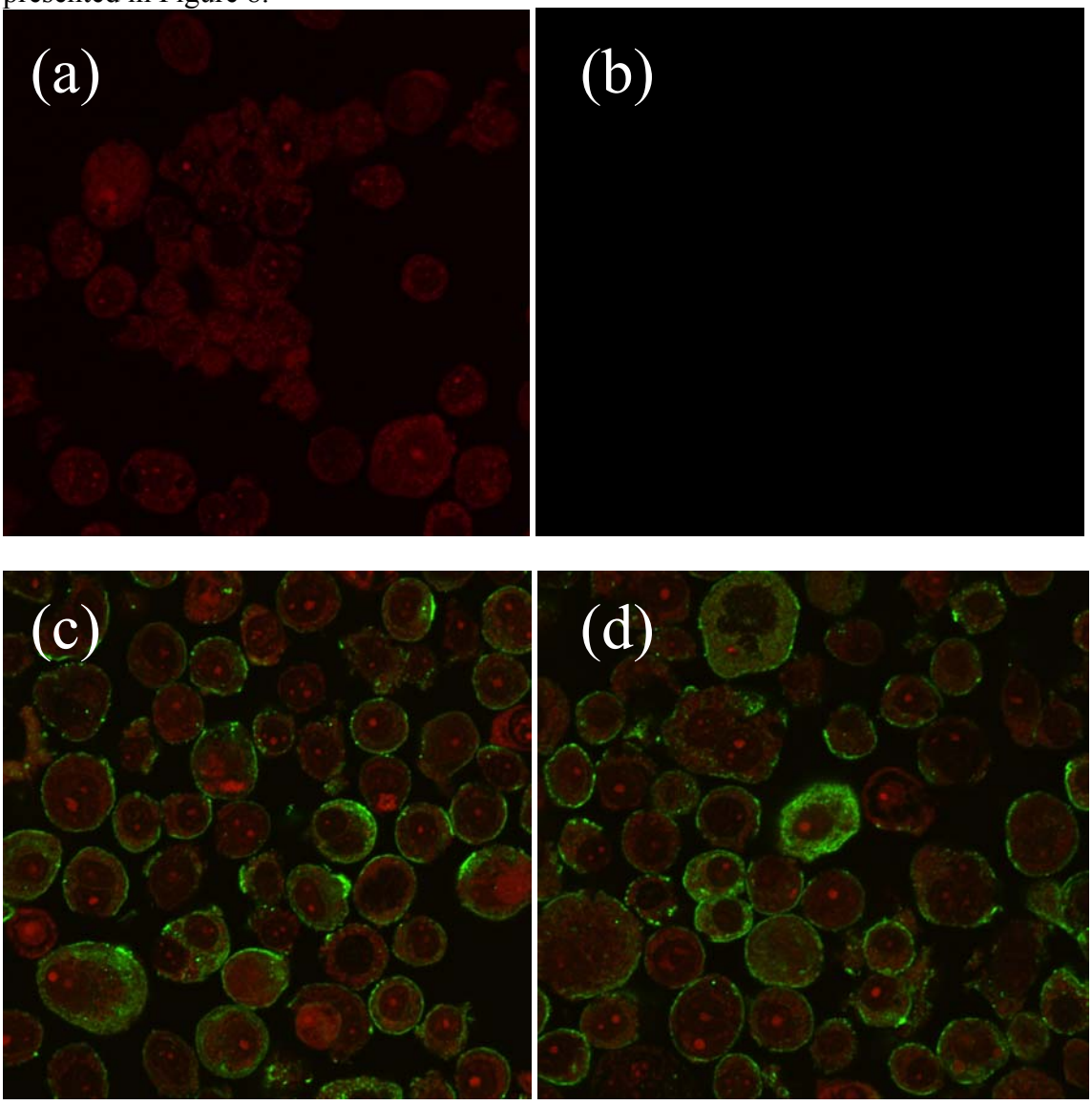

Figure 8: (a) Control group, SWCNT conjugated with Alexa Fluor 488 was completely terminated with PEG to prevent the binding with bio-molecules (no 1-pyrenebutanoyl succinimide was adsorbed on the side wall of the SWCNT). Such conjugate was mixed with complex Alexa Fluor 555 IGF1R and kept at room temperature for 30 minutes. MCF7 cancer

cells were again incubated with in such solution for 30 minutes. After which, the cells were triple rinsed and re-suspended to remove excessive fluorescent dyes. Under this configuration, almost no green signal could be detected on or inside the cells. (b) second control experiment where only SWCNT and Alexa fluor 488 was incubated with MCF7 cells for 30 minutes and confocal images showed diminished green color. (c) and (d) are the repeated experiments of the internalization of SWCNT.

Control studies using confocal microscopy indicated that when nanotubes that were terminated with PEG molecules to prevent binding with biomolecules and treated with IGF1R specific antibodies that were tagged with Alexa Fluor 555, and incubated with MCF7 cells, showed no green fluorescence on the cells illustrating no binding of SWNT on the membrane and no internalization (Figure 8a). In the second control experiment the cells were only incubated with SWCNT tagged with Alexa Fluor 488 and then micro-centrifuged to remove excess dye, triple rinsed and imaged using confocal microscopy. The resulting confocal images as presented in Figure 8 (b). The absence of green fluorescence demonstrated that nanotube by themselves do not get internalized within this period of time. Finally, SWNT tagged with Alexa 488 and IGF1R specific antibody tagged with Alexa 555 dye were mixed and kept at room temperature for 30 minutes. The suspension was then incubated with MCF7 cancer cells for 30 minutes, micro-centrifuged, rinsed and imaged using confocal microscopy. The resulting image is presented in Figure 8 c and Figure 8d. In these images one can clearly see the green fluorescence of nanotube both inside and on top of the cells. These results clearly indicate that nanotubes are internalized through the interaction of specific antibodies tagged to the nanotube surface with their corresponding antigens.

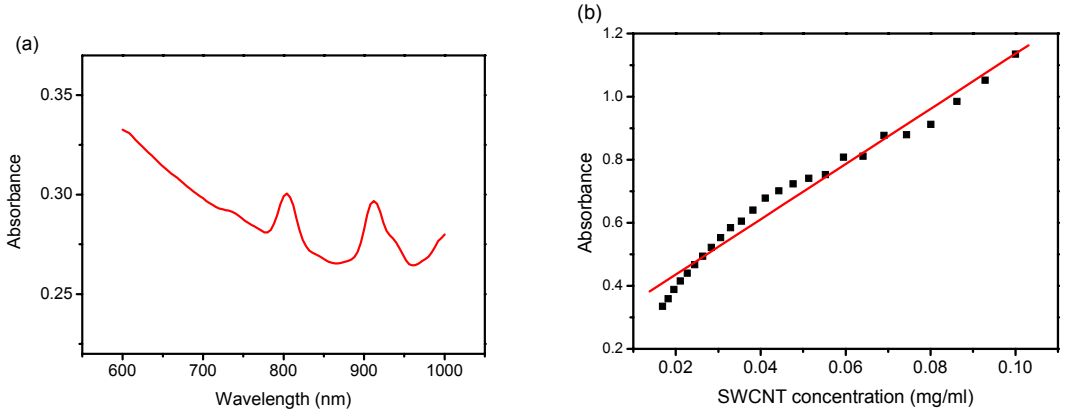

Figure 9 (a) Visible and near infrared spectra for 0.03 mg/ml SWCNT functionalized by IGF1R antibody. Peaks at ~808 nm and ~910 nm indicate the high absorbance of the complex in the NIR region. (b) absorbance of SWCNT of various concentrations in PBS solution. The solid line is the linear fit.

Optical properties of SWCNT were also examined in Figure 9. It was observed that over the NIR window, at about 808 nm wavelength, SWCNT-antibody conjugates have the highest absorbance. Such high absorbance leads to the application of NIR laser diode to transfer enough energy for cell destruction.

Figure 10 (a) shows the cells that were only treated by NIR dosing for 3 minutes and Trypan blue for investigating membrane permeability and cell death. As observed, Trypan blue did not

penetrate into the cell membrane and only can be seen on the background indicating no damage to the cell membrane. The cells that only received NIR light without internalized SWCNT survived after photon dosing showing high degree of transparency of biological systems to NIR light. In sharp contrast, in Figure 10 (b), the cells incubated with SWCNT-anti-Her-anti-IGF1 hybrids, were all bright blue, indicating membrane damage and cell death. We repeated these experiments several times and obtained similar results. Figure 11 is the repeat experiments.

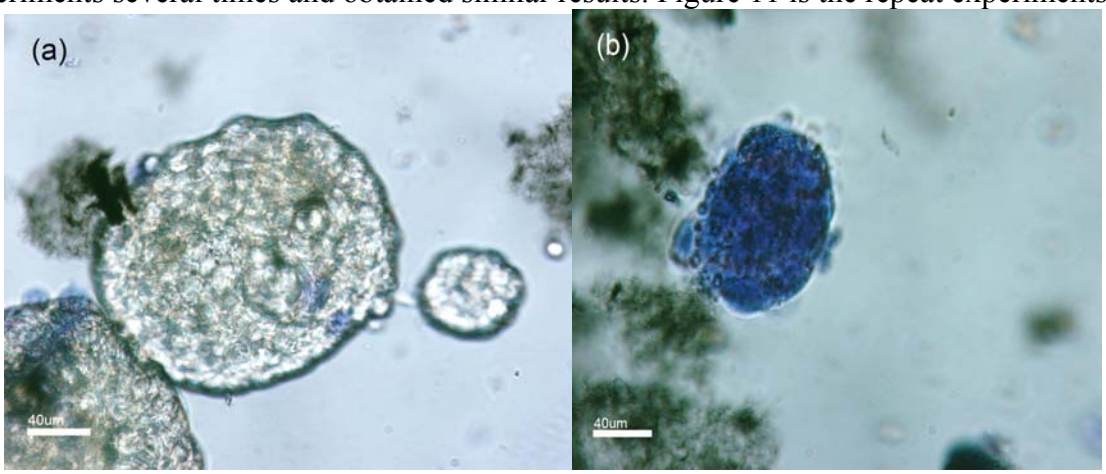

Figure 10 (a): MCF7 cancer cells treated without nanotube-nonspecific antibody complexes survived the NIR dosing at 800 mW/cm^2 for 3 minutes. Trypan blue was used to investigate membrane permeability and while the background looks blue, the cells appear white and reflective due to the impermeability of the Trypan blue. (b): MCF7 cancer cells treated with nanotube-anti-IGF1-Her2 antibody complex showing that all the cells died after NIR dosing at 800 mW/cm^2 for 3 minutes. The cells appear blue in color indicating cell death. Nanotubes start to precipitate as the samples started to dry around the cells.

Insets in Figure 10 and Figure 11 show the quantitative estimates of cell killing. It can be seen that cells treated with the non-specific hybrids then dosed with NIR survived better than the cells that received specific hybrids. This is hypothesized due to the limited binding effect between the complex and cell [26]. Collateral damage was observed in the non-specific experiments. Quantitative estimates were obtained by taking optical images of the cell clusters and placing a virtual 20 μm grid on top and count the number of dead cells in each grid from the circumference to the center of the cell. The insets indicate that targeting using non-specific antibodies resulted in some collateral damage. However, those collateral damages are mainly limited to the circumference of the cells and not the interior. Also in the circumferential region of the cell clusters targeted with non-specific antibodies, only 50% of the cells bonded to the nanotubes were observed to die following NIR dosing. The damages were limited and happened mainly on the circumference of the cell clusters due to sticky nature of the SWCNT that tend to bind to the cells more easily on the circumference. These collateral damages were also highly reproducible as seen in the insets of Figure 10 and 11 for non-specific and IGF1R and Her2 specific targeting. In a realistic sense, this is better than chemotherapy that tends to kill all the cells indiscriminately. However, even the IgG antibody can adhere to some cells during incubation and microcentrifugation can lead to collateral damage. The curvature of the SWCNT

after antibody binding and during cell incubation may also affect the surface adhesion of SWCNT to the cells.

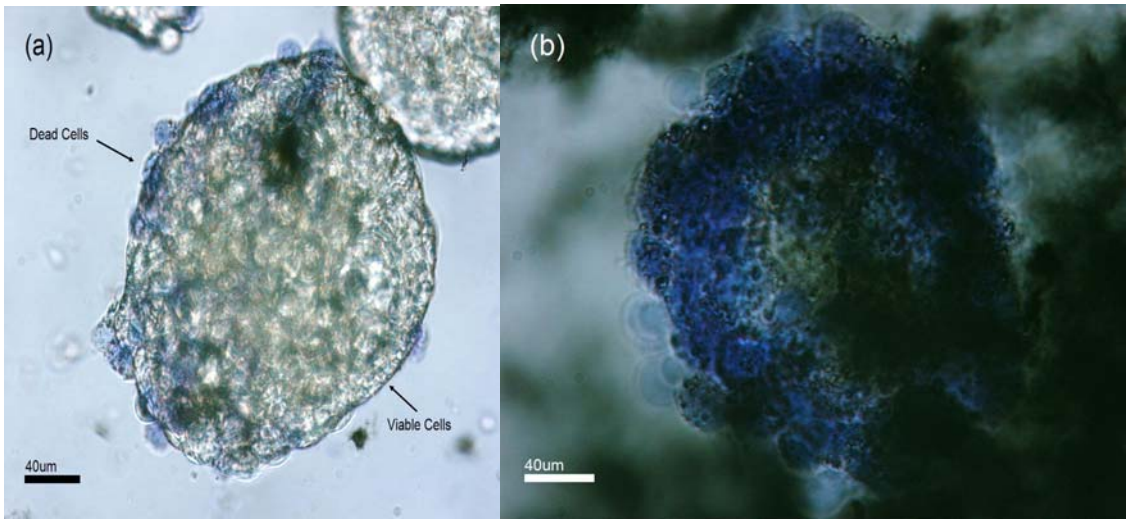

Figure 11 (a) MCF7 cancer cells treated with non-specific IgG antibody-nanotube complex did suffer from some damage as the collateral cells died after the laser excitation. Such limit killing effect was due to the unselective binding effect of conjugates. (b): repeated nanotube-anti-IGF1-Her2 antibody-nanotube complex MCF7 cancer cell destruction. Results show high repeatability of this experiment.

It may be reasonable to think that the cells may be acting as a suction pump for internalization of SWCNT. When antibodies attach to their corresponding receptors in cancer cells, stresses are generated due to the release in free energy and this may create pressure differences across the membrane pores thereby allowing the internalization of the SWCNT. This hypothesis is supported by previous work on bioassays of prostate specific antigens using micro-cantilevers [27, 28, 29]. Specific antibody-antigen binding creates much higher change in free energy compared to non-specific-antibody antigen binding [27]. This higher change in free energy can create large compressive stresses (large enough to bend a micro-cantilever [27]) on the surface of the receptor which in turn can expand the pores of the membrane thus allowing internalization of SWNT. Such large forces due to minimization of free energy can be utilized for transporting DNA, proteins and antibodies using nanoparticles, nanowires and nanotubes within minutes as evidenced in this study. Non-specific antibody-antigen binding also creates free energy change but the amount of change is almost an order of magnitude smaller. These could be some of the potential reasons for internalization of SWNT. However, studies on nanotube internalization require separate investigations into cellular uptake as a function of molecular binding, which can throw some light onto the intriguing properties of how cells internalize nanotubes. Such studies will also help us in better understanding of cellular processes in designing new types of drug delivery systems into the cells selectively. This study is also the first time to observe such internalization of SWCNT-antibody complexes over large areas of cells using receptor specific antibodies.

Antibodies incubated with the cells acted as biological transport carriers to realize the endocytosis of SWCNT. Shining NIR laser light heated the nanotubes inside the cells. The localized photothermal effect produced heat to destroy the cells completely. NIR was also used as it has been shown that cells are transparent to 700-1100 nm wavelength of light. Temperature

measurements of the samples with SWCNT dispersed in solution did not show more than 30°C increases for light intensities that are used here bringing the actual temperature of the sample to 55°C. This was confirmed by both thermocouples in solution as well as using infrared thermometers. However, the temperature changes can be dramatic if the SWCNTs are packed as that can trigger microscopic explosions in room environment when reacting with oxygen from room air [11]. The mechanism of light interaction on nanotubes is complex and poorly understood. Light causes interesting properties in nanotubes such as photo-conductivity due to exciton generation [30], light induced elastic deformation of nanotubes, electrostatic charge separation [31] and explosions such as SWCNT nanobombs [11]. These effects are highly important for biomedical applications. For example, the explosions can not only be used for cancer therapeutics but also to generate acoustic waves at the nanoscale for next generation of high efficiency ultrasound imaging applications. Stresses generated due to light on the surface of the SWCNT can also be used as a nanoscale delivery mechanism of proteins from the surface of the SWCNT into the cells.

The optical energy transduction into above mentioned energy domains can be high due to the optical absorbance. The high optical absorbance of SWCNT is a result of electronic transitions from the first or second van Hove singularities []. While the electron-phonon coupling is negligibly small in arm-chair type metallic nanotubes, they have been shown to be considerably high in zig zag semiconducting nanotubes. As a result the van Hove-like singularity in the density of states moves towards the top of the valance band enhancing the effective density of states near the Fermi energy [32]. This results in an increase in the electron-phonon interaction, thereby increasing the temperature of the nanotube. This also shows that semiconductive nanotubes are much better for applications in photo-dynamic therapy than metallic nanotubes. SWCNTs are supra-molecular structures and the molecular vibrations can be intense on light absorption. For applications in selective cancer therapy, more than actual temperature increases, the thermal gradients are also important so that heat is only generated on the cancer cells while the surrounding normal cells are maintained at 37°C. The thermal gradients in the nanoscale materials such as SWCNT can be extremely steep (~10^6-10^9 ºC/m). This means that while the nanotubes can heat up very locally to high temperatures, the steep thermal gradients localize the heating effects down from couple of nanometers to microns therefore resulting in highly localized cell killing effect as seen in our experiments.

These results show that by targeting surface receptors and NIR dosing, one can achieve a high degree of efficacy in destruction of cancer cells. While past studies have shown the utility of nanotubes to transport DNA and act as NIR agents to kill cells, this is the first study to show the multi-component targeting of more than one surface receptor (IGF1R and Her2) using SWCNT and simultaneous photo-dynamic therapy due to their high optical absorbance. The energy used in the destruction of cancer cells can be estimated to be ~200 nW per cell, too low to create any damage for the normal cells compared to past nanoparticle and nanotube based cell killing techniques [12, 16]. Past nanoparticle and nanotube based cell killing techniques have used 4-35 W/cm^2 and 1.4-3 W/cm^2 for 3-4 minutes producing temperature changes for cell killing. Our method uses half the laser power compared to past nanotube based cell killing technique and 5-44 times lower laser power compared to past nanoparticle technologies. High internalization of nanotubes into the cells therefore minimizes the amount of energy necessary for killing the cells.

Past nanotube studies have also only targeted folate receptor which is universally expressed in cells and nanoshells have targeted more breast cancer relevant Her2 surface receptor. We have

taken this a step further by showing multi-component targeting integrated with photo-dynamic therapy. By using nanostructures as biological transport carriers to target more than one surface marker, one can increase the efficiency of therapeutics that can lead to higher selectivity. Multi-component targeting and internalization followed by photothermal cell killing can therefore improve the efficiency and selectivity of therapeutics. Wide variety of proteins and molecules capable of overcoming biophysical barriers can be functionalized on the surface of the nanotubes as transport carriers. Nanotubes act as nanoscale thermal sources for material processing at the nanoscale and also for changing the kinetics of reactions of proteins inside cells. These could become building blocks of non-invasive future cell repair machines using nanotechnology and optics.

II. Preliminary Results on Integrated molecular imaging and photodynamic therapy in one step using gold nanoparticles and carbon nanotubes

The PI has generated encouraging preliminary results for multi-component molecular targeting of IGF1R and Her2 surface receptors and killing of breast cancer cells *in vitro* in one step. The hypothesis used here are as follows: Gold nanoparticles have been known to change color from red to blue when they aggregate in colloids. This is due to plasmon-plasmon interactions between locally adjacent nanoparticles and has been observed in the past [3] but never explored for direct cancer imaging. Similarly, carbon nanotubes show high optical absorbance in 700-1100 nm of electromagnetic spectrum [11,12]. Cells are transparent to 808 nm light and therefore won't be affected at lower light intensities and power. Exposed to 808 nm light, nanotubes can heat up to 55 C. This can be used for rupturing the cell membrane and killing the cancer cells while not harming the normal cells that may surround it. By combining gold nanoparticles and carbon nanotubes one can achieve simultaneous imaging and killing of cancer cells. Functionalizing the surface of the nanoparticles and nanotubes with IGF1R and Her2 specific antibodies can lead to improved targeting and killing of cells which can make therapeutics highly efficient. Figure 12 shows the in vitro preliminary results in this area.

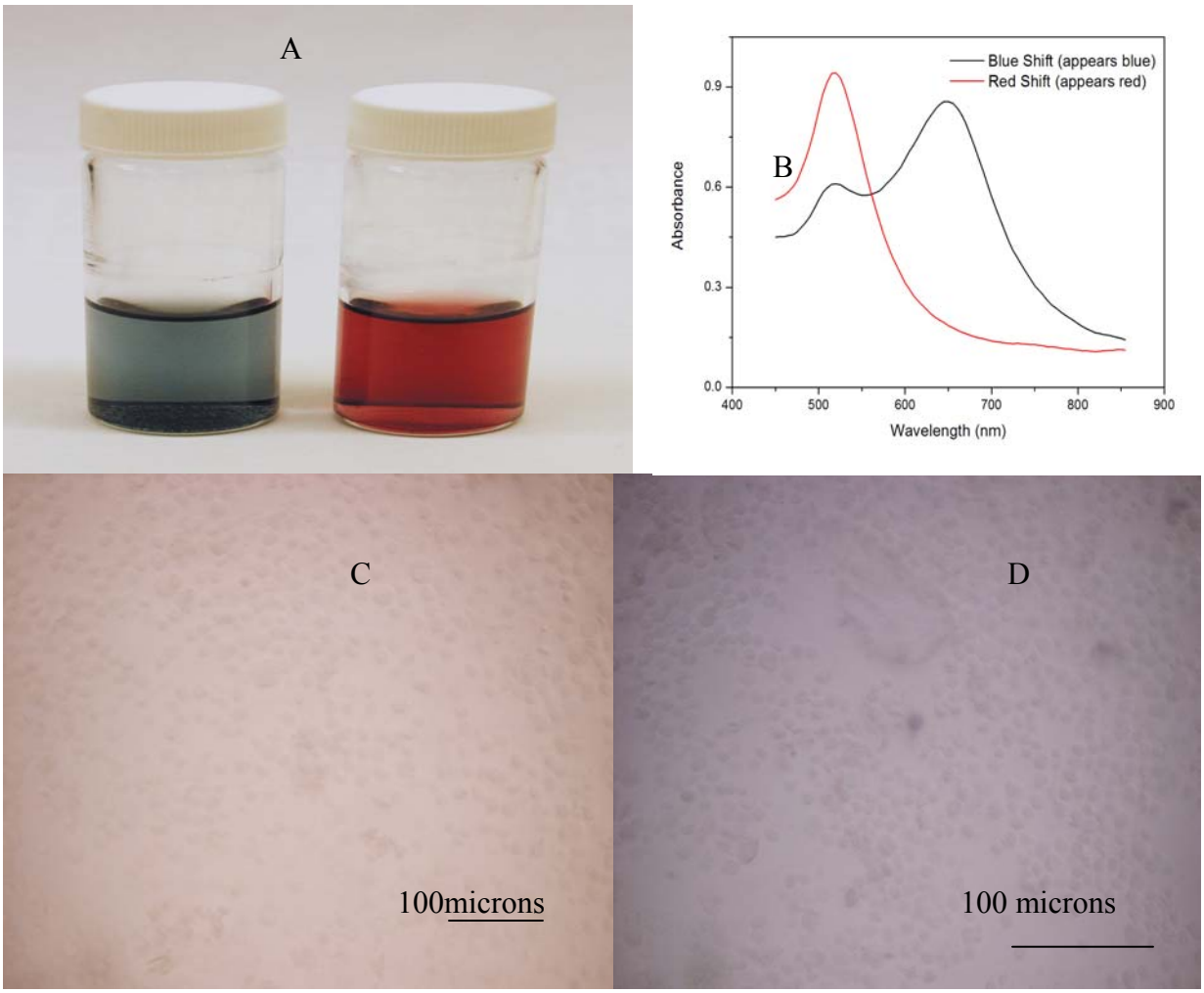

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Figure 12: (a) Aggregated gold nanoparticles (blue) and non-aggregated gold nanoparticles (red~ 5 nm) in solution, (b) shift in the optical absorption as a function of wavelength for gold nanoparticles, (c) MCF7 breast cancer cells incubated with gold nanoparticles in PBS and targeted to the IGF1R changed color from red (c) to blue (d) due to the aggregation of nanoparticles on the receptors,

Figure 12 is the spectrum of results that show the integrated approach of molecular targeting and cell killing in one step. Figure 3a demonstrates the visible change in color of aggregated and non-aggregated gold nanoparticles and Figure 3b shows the shift in optical absorbance of gold nanoparticles. To show the effectiveness of the color change with cells, the gold nanoparticles in solution were conjugated with the 1 mg/ml of IGF1R and 1 mg/ml of Her2 specific antibodies and incubated with the cells for an hour and imaged using optical microscope. After an hour the solution consisting of cells started to appear blue due to the docking of antibodies to their receptors and changing color due to the plasmon-plasmon effect. This simple but effective approach shows the feasibility of this concept.

With the no cost extension we can further investigate the endocytosis of gold nanoparticles and the resulting color change inside the cells. Further, confocal microscopy will be utilized to show the endocytosis of the nanoparticles using Alexa 488. For molecular targeting and cell killing,

first the carbon nanotubes and gold nanoparticles will be conjugated with both IGF1R and Her2 specific antibodies. Alexa 488 (green) and Alexa 555 (red) will be used for fluorescing the nanoparticles, and antibodies respectively for confocal microscopy. Non-specific antibodies will act as control groups in these experiments.

III Key Research Accomplishments

1. We have shown for the first time the integrated molecular targeting of receptors using monocolonal antibodies attached to carbon nanotubes and integrated photodynamic therapy.
2. High endocytosis of single wall carbon nanotubes over large areas was observed for the first time.
3. Targeting using IGF1R and Her2 specific antibodies resulted in high efficacy of cell killing using single wall carbon nanotubes.
4. The distance dependent color changing property of gold nanoparticles has been used for the first time to investigate molecular overexpressions in breast cancer cells using optical microscopy.
5. A paper demonstrating this technique was published recently in Nanotechnology. This is a cover article and is featured in the August issue of Nanotechnology.

IV. Reportable Outcomes:

The following are the reportable outcomes of this award:

- Several manuscripts have been lined up that illustrates the utility of nanotubes and gold nanoparticles for breast cancer research
- A presentation was given at the Nanotech 2007 conference on Integrated Molecular Targeting and Photodynamic Therapy using Carbon Nanotubes in May 2007 in California.
- The PI serves on the editorial board of the Journal Nanobiotechnology that caters directly to the medical applications of nanotechnology. This wouldn't have been possible with out the dissemination of the current results.
- A student Ning Shao has obtained his Masters degree due to this research.
- An NIH grant (R22/R33) has been applied from the results generated through this award for further study in animals and human subjects.
- Collaborations with medical schools such as Thomas Jefferson University has been strengthened due to this award with Prof. Eric Wickstrom.

V Conclusions

In this project, we have demonstrated multi-component molecular targeting of surface receptors (IGF1R and Her2) and NIR dosing of cancer cells using SWCNT. While previous studies have shown the transport of DNA into cells using nanotubes, in this study we show multi-component molecular targeting of both IGF1R and Her2 surface markers in cancer cells using single wall carbon nanotubes. IGF1 and Her2 specific antibodies conjugated to the SWCNT were used to target their corresponding receptors in cells and internalize the SWCNT. The cells were then dosed with NIR 808 nm photons at ~800 mW/cm^2 for 3 minutes. Cells that were treated with non-specific antibody-SWCNT hybrids survived the NIR dosing compared to the dead cells with anti-IGF1-anti-Her2-SWCNT hybrids. The amount of energy consumed for cell killing was estimated to be as small as ~200 nW per cell, which is an order of magnitude smaller than competing techniques. Quantitative estimates showed that cells incubated with SWCNT-anti-Her-anti-IGF1R antibodies were completely destroyed while 80% of the non-specific-antibody-SWCNT hybrids treated cells were still alive. These results indicate that SWCNT could be used as biological transport agents and the high optical absorbance in the NIR are capable of killing cancer cells with minimum collateral damage. Further, western blots indicated the relevance of targeting IGF1R in MCF7 cancer cells and Her2 in BT474 breast cancer cells. This study is another clear example of molecular targeting and cell killing approach that can improve therapeutic efficacies. These tools can also be applied to traditional surgical oncology to improve the probability of killing cancer cells whose microscopic foci are often left behind in surgery.

With the no cost extension we will be able to finish the work on conjugating gold nanoparticles with carbon nanotubes and show the integrated molecular targeting of IGF1R and Her2 surface receptors in breast cancer cells that will help in imaging and therapy in one step. The continuation of this grant will allow us to study the effect of cell interactions on antibodies coated on nanotubes and gold nanoparticles, measure their optical properties and also investigate cell killing using nanotubes by heating the nanotubes using infra-red light. The potential applications of this study are enormous as this is the first time to conclusively show the molecular targeting, imaging and therapy all in one step. Further, the optical fluorescence of nanotubes can be utilized to image cells and tissues at the sub-100 nm limits using conventional microscopic techniques. This study also shows the utility of nanotubes as contrast agents for high resolution sub-micron to sub-100 nm MRI imaging which is not possible using present day technology. The potential applications of nanotubes for cancer research are therefore enormous.

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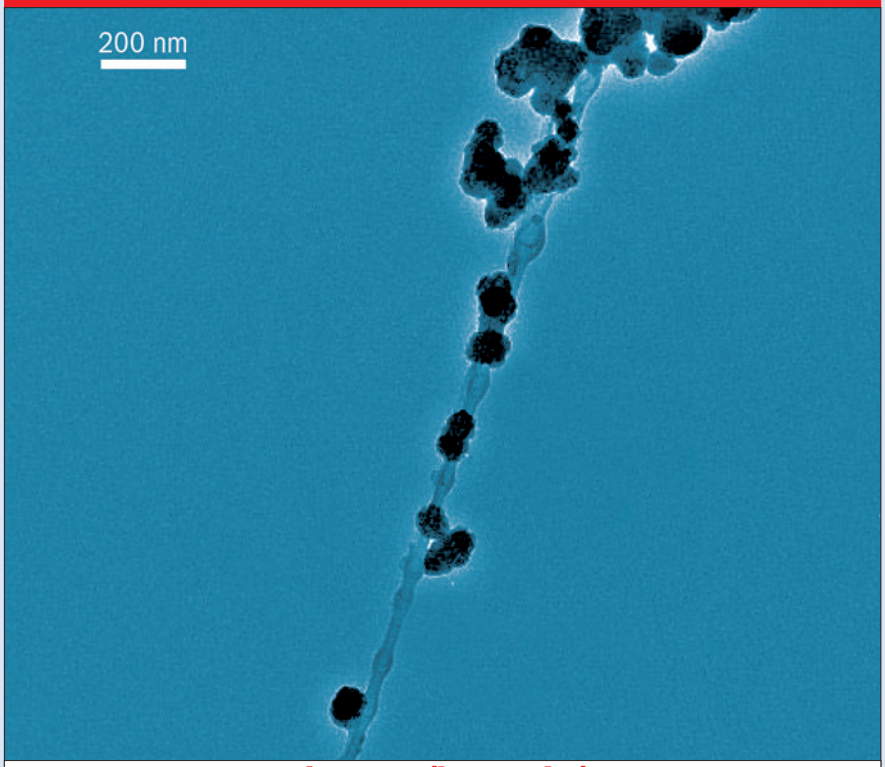

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Integrated molecular targeting of IGF1R and HER2 surface receptors and destruction of breast cancer cells using single wall carbon nanotubes

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Abstract
Molecular targeting and photodynamic therapy have shown great potential for selective cancer therapy. We hypothesized that monoclonal antibodies that are specific to the IGF1 receptor and HER2 cell surface antigens could be bound to single wall carbon nanotubes (SWCNT) in order to concentrate SWCNT on breast cancer cells for specific near-infrared phototherapy. SWCNT functionalized with HER2 and IGF1R specific antibodies showed selective attachment to breast cancer cells compared to SWCNT functionalized with non-specific antibodies. After the complexes were attached to specific cancer cells, SWCNT were excited by ∼808 nm infrared photons at ∼800 mW cm^{-2} for 3 min. Viability after phototherapy was determined by Trypan blue exclusion. Cells incubated with SWCNT/non-specific antibody hybrids were still alive after photo-thermal treatment due to the lack of SWNT binding to the cell membrane. All cancerous cells treated with IGF1R and HER2 specific antibody/SWCNT hybrids and receiving infrared photons showed cell death after the laser excitation. Quantitative analysis demonstrated that all the cells treated with SWCNT/IGF1R and HER2 specific antibody complex were completely destroyed, while more than 80% of the cells with SWCNT/non-specific antibody hybrids remained alive. Following multi-component targeting of IGF1R and HER2 surface receptors, integrated photo-thermal therapy in breast cancer cells led to the complete destruction of cancer cells. Functionalizing SWCNT with antibodies in combination with their intrinsic optical properties can therefore lead to a new class of molecular delivery and cancer therapeutic systems.

1. Introduction

Despite outstanding progress in the area of cancer biology, significant challenges remain in administering highly selective, targeted anti-cancer therapy. It is striking to note that only 1–10 parts per 100 000 intravenously administered monoclonal antibodies reach their parenchymal targets *in vivo* [1]. Nanoparticles [2, 3], nanoshells [4, 5] and nanotubes [6, 7] have been shown in the past to be quite applicable for cancer imaging and therapy. Subcellular

nanostructures exhibiting advanced physical properties for biological applications can have an important impact on the introduction and delivery of DNA, proteins and drug molecules into living cells [7]. In addition, the unique electronic [8, 9], optical [10, 11] and thermal properties [12] and the subcellular size of nanostructures makes them very attractive for multi-component targeting of surface receptors to enhance the efficacy of anti-cancer agents.

Nanoscale materials with opto-thermal transduction principles have been shown to be an interesting approach for the destruction of cancer cells. Nanotubes are very suitable for this technique because of their high efficiency in optical absorbance and photo-thermal cell destruction [7]. The amount of light energy used is quite small compared to competing techniques as the optical absorbance of nanotubes is high in the 700–1100 nm near-infrared (NIR) window. This has recently been used to demonstrate many new types of optical sensors and actuators based on the photo-mechanical actuation of carbon nanotubes [13–15]. Therefore multi-component targeting with photo-thermal cell destruction might lead to the development of cancer therapeutics, micro-surgery and cell repair machineries.

While nanoshells have been targeted to the surface receptor of cancer cells in the past [4], in this report we show the integrated targeting of both insulin-like growth factor 1 receptor (IGF1R) and human endothelial receptor 2 (HER2) surface receptors and photo-thermal cell destruction using single wall carbon nanotubes (SWCNT). Multi-component targeting of more than one surface receptor can lead to higher efficacies in therapeutics. Past studies involving SWCNT have targeted the folate receptors in folate positive cancer cell studies [7]. In contrast, IGF1R and HER2 are both relevant surface markers that are overexpressed in a wide variety of cancer cells compared to normal cells and therefore have great potential for sensing, imaging and therapy of cancer. Circulating cancer cells often express characteristic cell surface markers. Human MCF7 ER+ breast cancer cells exhibit high expression of IGF1R, while BT474 ER− cancer cells have lower expression of IGF1R but high expression of HER2 [16].

2. Experimental details

2.1. Preparation of SWCNT solution

1 mg ml^{-1} of SWCNT (Nano-Lab, lot number FH-P 071706) in phosphate buffered saline solution (PBS) was agitated for 1 h under room temperature in an ultrasonicator (Fisher Scientific FS60H) to break the SWCNT bundles into individual nanotubes. The solution was then micro-centrifuged at 10 000 × *g* for 15 min. Sediments containing mostly large conjugations of SWCNT were discarded and supernatants were collected. To closely examine the dispersion of SWCNT, samples were prepared for transmission electron microscopy (TEM) by dropping 1 μl of such solution on top of a TEM grid. TEM images of the SWCNT were taken from different parts of the solution to ensure that SWCNT were uniformly dispersed within the solution.

2.2. Antibody functionalization of SWCNT

Five milligrams of 1-pyrenebutanoyl succinimide was dissolved in 50 ml of methanol with gentle agitation at room temperature. One millilitre of solution was mixed with 1 ml SWCNT solution in phosphate-buffered saline (PBS) and kept at 20 °C for 30 min to allow the reaction between the reactants. Five milligrams of polyethylene glycol (PEG) (Acros Organic, MW 8000) was diluted in the solution to form a self-assembly monolayer (SAM) on unoccupied sites so that the SWCNT were insulated from outside liquid to prevent undesirable binding with other biomolecules. The already functionalized SWCNT were then filtered, collected, triple rinsed, re-suspended in PBS and kept at 4 °C for storage. Such a protocol ensures adhesion of SWCNT and 1-pyrenebutanoyl succinimide, the removal of excess reagents, and allows the complex to be stored for long periods. Non-specific mouse anti-human myeloma immunoglobulin G (IgG), anti-IGF1R mouse monoclonal antibody and anti-HER2 (Merck Bioscience, Calbiochem Inc.) were prepared by diluting 1 mg ml^{-1} monoclonal antibody solution with PBS. Following this, 1 ml of SWCNT solution was mixed with 1 ml of monoclonal antibody solution (IGF1R or HER2) and the solution was allowed to incubate for 1 h at room temperature and micro-centrifuged. The sample was triple rinsed to remove excess antibodies in the incubation liquid. These procedures yielded two different solutions containing SWCNT–anti-IGF1R antibody and SWCNT–anti-HER2 antibody conjugates. Samples for microscopy were then prepared by dropping a small droplet on top of the TEM grids and several samples were investigated for antibody functionalization using TEM to ensure the repeatability of the antibody-adsorbing effect.

2.3. Toxicity of functionalized single wall carbon nanotubes

Since cancer cell killing techniques may be applicable *in vivo*, it is important to understand the toxicity of nanotubes. Nanotubes were claimed to be toxic after injection of 15 mg ml^{-1} into the lungs of mice, which died within a week of injection due to asphyxiation [17]. Nanotubes belong to the same family as fullerenes and fullerene derivatives have been shown to be non-toxic for medical applications. Recently water-soluble nanotubes have been shown to be non-toxic and there are data indicating that nanotube derivatives are non-toxic too [18]. By chemically modifying the surface of nanotubes with phenyl, COOH and OH groups nanotubes have been shown to be non-toxic; furthermore, optical and atomic force microscopy show direct contact between cellular membranes and water-dispersible SWCNTs [18]. Our experiments on the preparation of SWCNT initially with PBS and 1-pyrenebutanoyl succinimide in methanol are expected to have plenty of OH and COOH groups that make them less toxic. The presence of PEG to inhibit non-selective attachment also reduces the toxicity of nanotubes greatly due to the presence of OH groups.

To examine the potential toxicity of SWCNT a cell viability assay was performed. MCF7 cells were incubated with functionalized nanotubes using the same procedure mentioned above in section 2.2. Five milligrams of 1-pyrenebutanoyl succinimide was dissolved in 50 ml of methanol with gentle agitation under room temperature. One millilitre of solution was mixed with 1 mg ml^{-1} SWCNT solution in PBS and kept at 20 °C for 30 min to allow the reaction between the reactants. Five milligrams of PEG (Acros

Organic, MW 8000) was diluted in the solution to form a SAM on unoccupied sites so that the SWCNT were insulated from outside liquid to prevent undesirable binding with other biomolecules. The functionalized SWCNT were then filtered, collected, triple rinsed and re-suspended in PBS. MCF7 cells were incubated with the functionalized nanotubes, isolated by centrifugation and observed after 48 h. MCF7 cells without nanotubes acted as control experiments. Following incubation for 48 h, Trypan blue dye was used to investigate cell viability.

2.4. Fluorescent SWCNT–antibody hybrids

300U Alexa Fluor 488 phalloidin and 555 (Invitrogen) were dissolved in 1.5 ml methanol and kept at −20 °C for storage. Ten millilitres of SWCNT in PBS at 0.1 mg ml^{-1} was mixed with 10 μl Alexa Fluor 488 to deliver a green colour under confocal laser excitation. Similarly, 40 μl pure antibody solutions at 1 mg ml^{-1} were dyed red with 10 μl Alexa Fluor 555. The two mixtures were kept at room temperature for 30 min to allow the optimum reaction. The two solutions were mixed following previously mentioned protocol to form Fluor 488–SWCNT–antibody–Fluor 555 complexes for further cell endocytosis.

In order to remove the excessive fluorescent dyes the solution was micro-centrifuged at 10 000 × *g* for 5 min. The bottom solution containing mostly the heavy Fluor 488–SWCNT–antibody–Fluor 555 conjugates was collected and re-suspended in PBS.

2.5. Cell culture and incubation with SWCNT

Human BT474 and MCF7 breast cancer cells (ATCC, Germantown, MD) were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 5000 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 10% fetal bovine serum at 37 °C under 5% CO_2 for 48 h before experiments. MCF7 cells were further supplemented with 7.5 nm 17-β-estradiol (Sigma). Cells released by ethylenediaminetetraacetic acid (EDTA) were micro-centrifuged at 100 × *g* for 5 min. Precipitates containing the cells were collected and re-suspended in PBS. Four μl of SWCNT–anti-IGF1R antibody solution was mixed with 4 μl of cells and incubated for 1 h. Following this, 4 μl of this solution was then mixed with the same amount of SWCNT–anti-HER2 antibody solution, incubated for 1 h and then micro-centrifuged. This protocol ensured multi-component targeting of both IGF1R and HER2 before photo-thermal dosing of cancer cells. SWCNT–anti-IgG non-specific hybrids were obtained through similar procedures.

Two control experiments were investigated to show binding of SWCNT to the cell membrane and internalization through receptor specific antibodies. In one set of experiments, nanotubes were terminated with PEG to prevent binding of biomolecules (1-pyrenebutanoyl succinimide was not adsorbed on the side wall of SWCNT which enables binding of antibodies). Such conjugate was mixed with IGF1R specific antibodies that were tagged to Alexa Fluor 555. The resulting suspension was then incubated with MCF7 cells for 30 min. In another set of experiments, SWCNT was conjugated with Alexa Fluor 488 and incubated with MCF7 cells directly for 30 min. The resulting suspension was micro-centrifuged, rinsed and imaged using confocal microscopy.

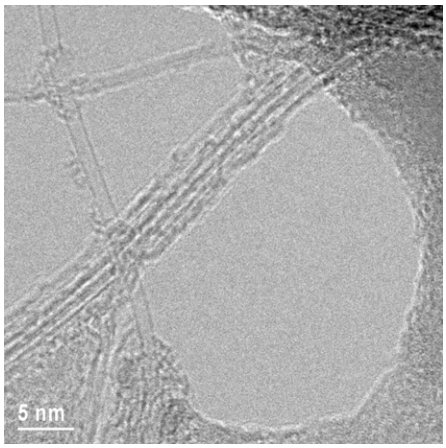

Figure 1. Transmission electron micrograph of single wall carbon nanotube about 1.4 nm in diameter. The single-layer sidewall is clearly visible.

2.6. Photo-thermal therapy

Following incubation of SWCNT–antibody complex with the cancer cells, laser light of 808 nm at 800 mW cm^{-2} was dosed for 3 min to all the samples. After laser excitation, membrane permeability was investigated by adding 0.4% Trypan blue in PBS. Optical microscopy was used to record the images of cell viability, membrane permeability and nanotube binding. The lasers used were tunable from 50 mW cm^{-2} to 1 W cm^{-2} and intensities were calibrated by power meters. A custom built circuit was used for closed loop control of the lasers. The laser spot size was about 1 cm for the photo-thermal dosing of samples, which ensured that all the cells in the titre plate received uniform photon dosing.

3. Results

The SWCNT were about 1.4 nm in diameter and the typical length varied from 500 nm to 1 μm (figure 1). The shorter SWCNT tend to be straighter with fewer curves which makes endocytosis easier. The functionalization scheme of antibodies is shown in figure 2. This procedure worked very well for attachment of both specific and non-specific antibodies on the nanotube surface. TEM of the antibody functionalized SWCNT gives us direct confirmation that the antibodies were attached to the SWCNT on many different sites, as imaged in figure 3. This has also been confirmed in the past using atomic force microscopy (AFM) and confocal microscopy [19]. Potentially each group of carbon atoms equal to the cross-sectional area of the constant region of a monoclonal antibody (mAb) constitutes a binding site in the SWCNT that can act as a biological carrier to transport macromolecules inside the cells. Past AFM studies have shown nanotube–antibody hybrids to be 5–8 nm high [19]. Subtracting the diameter of the nanotube ∼1.4 nm, this makes the effective height of the antibodies atop the nanotube ∼3–6 nm. From TEM images presented in figure 3, it can be observed that each one of the antibody aggregates varies between 66 and 100 nm. Hence it is expected that about 10–25 antibody molecules per site have been aggregated. Since the

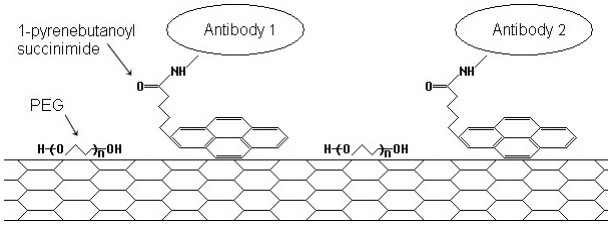

Figure 2. Functionalization schematic of single wall carbon nanotubes with monoclonal antibodies. 1-pyrenebutanoyl succinimide was adsorbed on the nanotube through π–π stacking. The succinimidyl ester can react with available lysine sidechain amines on antibodies to form amide bonds. PEG acts as an insulator to prevent potential bio-fouling.

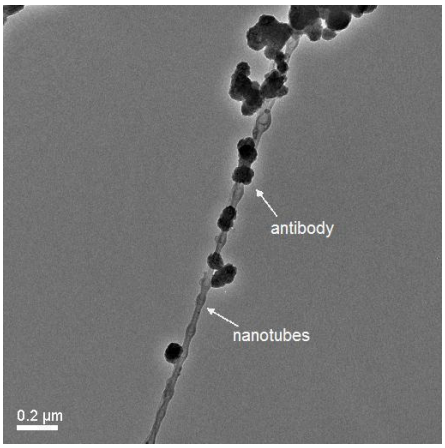

Figure 3. Transmission electron micrograph of the functionalization of monoclonal antibody aggregates specific to IGF1 receptor in breast cancer cells. Each site consists of 10–25 antibody molecules.

nanotubes conjugated with antibodies were rinsed three times before imaging, it is expected that all the antibody aggregates are covalently bonded to the carbon nanotubes. This shows that a greater number of antibody molecules per site can be attached to SWCNT without loss of activity for drug delivery.

One of the most intriguing observations that we made is how the SWCNT become internalized into the cells. Optical and confocal images obtained after the incubation of SWCNT–antibody conjugates with the cells demonstrated that the SWCNT were readily internalized into the cells over large areas, as shown in figure 4. The dark areas seen on the optical images were regions of internalized SWCNT. Trypan blue-excluding cancerous cells were also optically imaged as control group.

A cell viability assay was performed and the results are shown in figure 5. MCF7 cells incubated with SWCNT for 48 h exhibited little toxicity. The groups containing nanotubes exhibited about 10% cell death and the control group without nanotubes exhibited about 5% cell death. Most of the cells in both these groups are viable, as can be seen by the lack of penetration of Trypan blue dye into the cell membrane. While some cells in the nanotube group show a faint blue colour, these are on the surface of the cells and do not penetrate into the cell membrane, showing the viability of these cells treated with nanotubes. These results show that functionalized nanotubes themselves exhibit little toxicity to breast cancer cells under study.

It should be noted that human MCF7 ER+ breast cancer cells exhibit high expression of IGF1R, while BT474 ER− cancer cells have lower expression of IGF1R but high expression of HER2. While there are several studies that show the overexpression of HER2 in BT474 cells [20], western blot analysis was performed to show the overexpression of IGF1R in MCF7 versus BT474 breast cancer cells. This result is presented in figure 6. It can be observed that MCF7 breast cancer cells have higher overexpression of IGF1R, which is therefore concluded to be a relevant surface marker for molecular targeting.

Cancer cells incubated with fluorescent specific antibody–SWCNT complex were imaged by confocal microscopy in figure 7. Images were obtained at the same spot but using different filters. SWCNT functionalized with Alexa Fluor 488 appeared green inside the cell and on the edge of the cell membrane while Alexa Fluor 555 delivered a red colour to the antibodies and was observed mostly inside the cells. The less intense green fluorescence was caused by the limited binding effect of the dye and SWCNT. Curvature of the SWCNT also made them conjugate on the cell membrane which was confirmed by the bright green edge of the cells. Staining of cells with internalized SWCNT with Trypan blue did not indicate cell death after incubation. No difference in the internalization was observed for IGF1R and HER2 specific antibodies that were functionalized on the surface of the SWCNT. This process is also continuous, and with long periods (>30 min) of time the SWCNT become highly internalized into the cells due to the binding of the antibodies to their corresponding receptors. Non-specific antibodies did not show such internalization but were attached to the circumference of the cell clusters. More studies on the internalization of nanotubes with non-specific and IGF1R and HER2 specific antibodies showed clear internalization of specific antibody nanotube complexes into the cells. This is presented in figure 8.

Control studies using confocal microscopy indicated that when nanotubes that were terminated with PEG molecules to prevent binding with biomolecules and treated with IGF1R specific antibodies that were tagged with Alexa Fluor 555, and incubated with MCF7 cells, they showed no green fluorescence on the cells illustrating no binding of SWCNT on the membrane and no internalization (figure 8(a)). In the second control experiment the cells were only incubated with SWCNT tagged with Alexa Fluor 488 and then micro-centrifuged to remove excess dye, triple rinsed and imaged using confocal microscopy. The resulting confocal images are presented in figure 8(b). The absence of green fluorescence demonstrates that nanotubes by themselves do not become internalized within this period of time. Finally, SWCNT tagged with Alexa 488 and IGF1R specific antibody tagged with Alexa 555 dye were mixed and kept at room temperature for 30 min. The suspension was then incubated with MCF7 cancer cells for 30 min, micro-centrifuged, rinsed and imaged using confocal microscopy. The resulting image is presented in figures 8(c) and (d). In these images one can clearly see the green fluorescence of nanotubes both inside and on top of the cells. These results clearly indicate that nanotubes are internalized

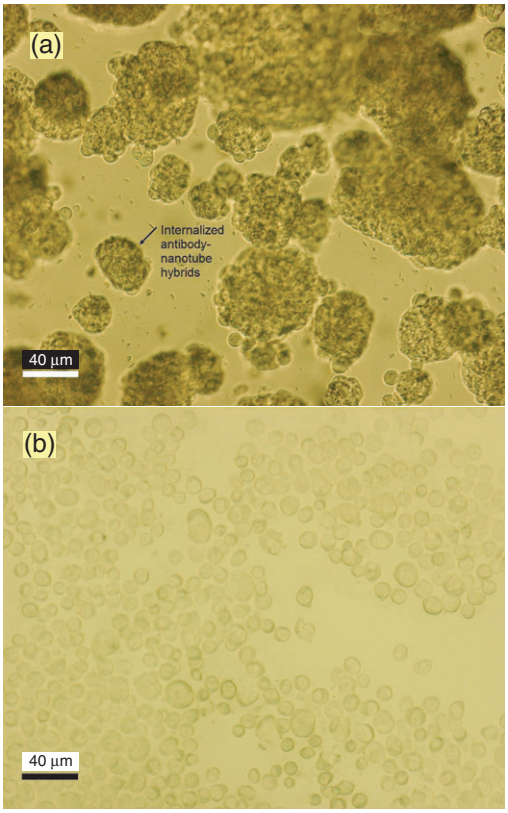

Figure 4. (a) Internalization of IGF1R–nanotube hybrid into MCF7 breast cancer cells. The dark regions are regions of internalized nanotube–antibody hybrids. (b) Control group of cancer cells without SWCNT internalization.

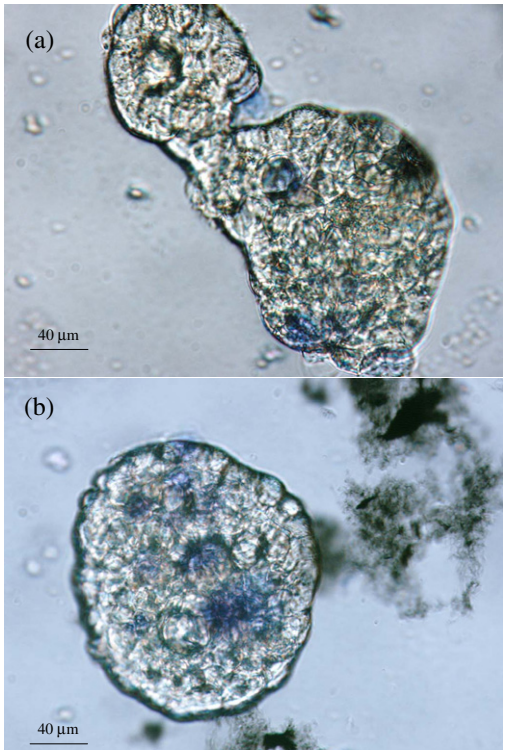

Figure 5. Cell viability assay: (a) control group of MCF7 cancer cells without functionalized nanotubes, (b) MCF7 cancer cells incubated with functionalized SWCNT for 48 h followed by Trypan blue exclusion.

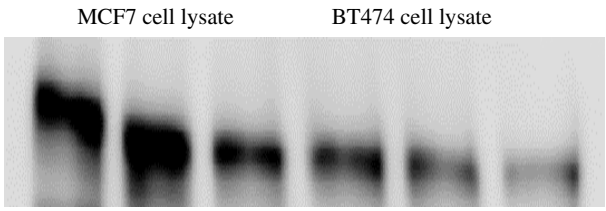

Figure 6. Western blots of IGF1R in BT474 versus MCF7 cells showed a band ratio of 0.466 ± 0.241 ($n = 6$, relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)).

through the interaction of specific antibodies tagged to the nanotube surface with their corresponding antigens.

Optical properties of the SWCNT were also examined in figure 9. It was observed that over the NIR window, at about 808 nm wavelength, SWCNT–antibody conjugates have the highest absorbance. Such a high absorbance leads to the application of a NIR laser diode to transfer enough energy for cell destruction.

Figure 10(a) shows the cells that were only treated by NIR dosing for 3 min and Trypan blue for investigating membrane permeability and cell death. As observed, Trypan blue did not penetrate into the cell membrane and can only be seen on the background indicating no damage to the cell membrane. The cells that only received NIR light without internalized SWCNT survived after photon dosing, showing a high degree of transparency of biological systems to NIR light. In sharp contrast, in figure 10(b), the cells incubated with SWCNT–anti-HER–anti-IGF1 hybrids were all bright blue, indicating membrane damage and cell death. We repeated these experiments several times and obtained similar results. Figure 11 shows the repeat experiments.

Insets in figures 10 and 11 show the quantitative estimates of cell killing. It can be seen that cells treated with the non-specific hybrids then dosed with NIR survived better than the cells that received specific hybrids. This is hypothesized to be due to the limited binding effect between the complex and cell [21]. Collateral damage was observed in the non-specific experiments. Quantitative estimates were obtained by taking optical images of the cell clusters and placing a virtual 20 μm grid on top and counting the number of dead cells in each grid from the circumference to the centre of the cell. The insets indicate that targeting using non-specific antibodies resulted in some collateral damage. However, the collateral damage is mainly limited to the circumference of the cells and not the interior. Also in the circumferential region of the cell clusters targeted with non-specific antibodies, only 50% of the cells bonded to the nanotubes were observed to die following NIR dosing. The damage was limited and happened mainly on the circumference of the cell clusters due to sticky nature of the SWCNT that tend to bind to the cells more easily on the circumference. This collateral damage was also highly reproducible as seen in the insets of figures 10 and 11 for non-specific and IGF1R and HER2 specific targeting. In a realistic sense, this is better than chemotherapy that tends to

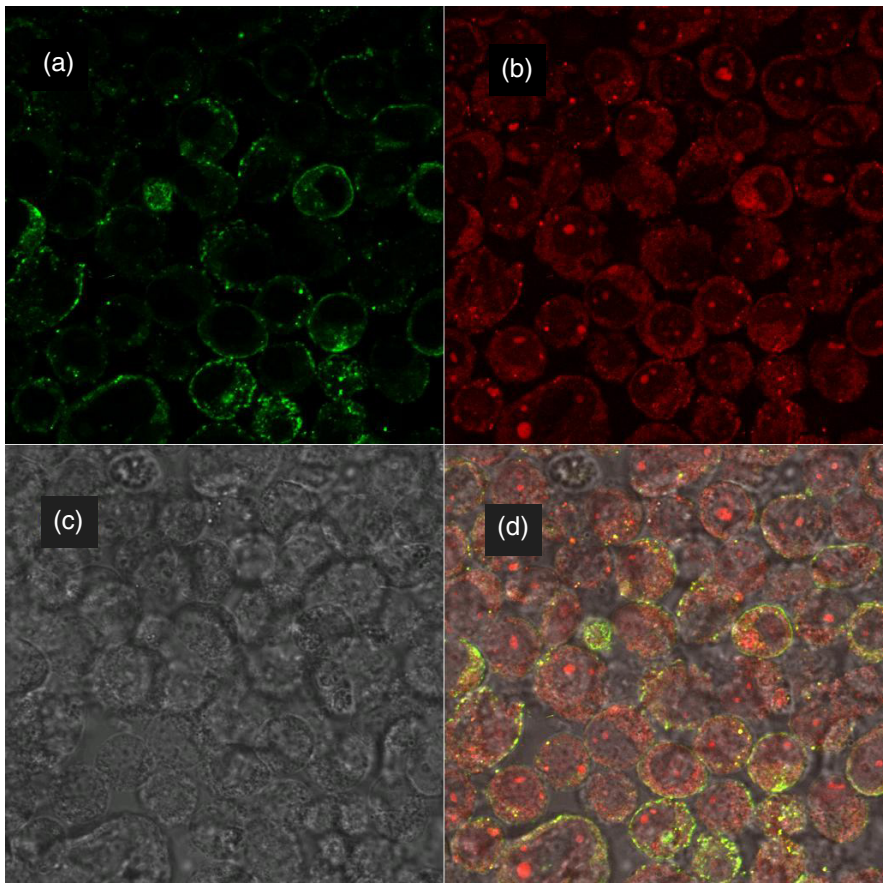

Figure 7. Confocal microscopy of specific antibody-facilitated SWCNT internalization of MCF7 cancerous cells. SWCNT and IGF1R antibody were tagged with Alexa Fluor 488 and Alexa Fluor 555 dye, respectively. The green (a) and red (b) are fluorescent SWCNT and antibody, respectively. By overlapping (a), (b) and optical image (c), the facilitated SWCNT endocytosis could be confirmed by image (d). Scale bar: 10 μm.

kill all the cells indiscriminately. However, even the IgG antibody can adhere to some cells during incubation and micro-centrifugation can lead to collateral damage. The curvature of the SWCNT after antibody binding and during cell incubation may also affect the surface adhesion of SWCNT to the cells.

4. Discussion

It is reasonable to think that the cells may be acting as a suction pump for internalization of SWCNT. When antibodies attach to their corresponding receptors in cancer cells, stresses are generated due to the release of free energy and this may create pressure differences across the membrane pores, thereby allowing the internalization of the SWCNT. This hypothesis is supported by previous work on bioassays of prostate specific antigens using micro-cantilevers [22, 23]. Specific antibody–antigen binding creates much higher change in free energy compared to non-specific-antibody–antigen binding [22]. This higher change in free energy can create large compressive stresses (large enough to bend a micro-cantilever [22]) on the surface of the receptor which in turn can expand the pores of the membrane thus allowing internalization of SWCNT. Such large forces due to minimization of free energy can be utilized for transporting DNA, proteins and antibodies using nanoparticles, nanowires and nanotubes within minutes, as evidenced in this study. Non-specific antibody–antigen binding also creates a free energy change but the amount of change is almost an order of magnitude smaller. These could be some of the potential reasons for internalization of SWCNT. However, studies on nanotube internalization require separate investigations into cellular uptake as a function of molecular binding, which can throw some light onto the intriguing properties of how cells internalize nanotubes. Such studies will also help us to better understand the cellular processes needed for designing new types of drug delivery systems into the cells selectively. This study is also the first to observe such internalization of SWCNT–antibody complexes over large areas of cells using receptor specific antibodies.

Antibodies incubated with the cells acted as biological transport carriers to realize the endocytosis of SWCNT. Shining NIR laser light heated the nanotubes inside the cells. The localized photo-thermal effect produced heat to destroy the cells completely. NIR was also used as it has been shown that cells are transparent to the 700–1100 nm wavelength of light. Temperature measurements of the samples with SWCNT dispersed in solution did not show more than $30 \pm 7\,^{\circ}\mathrm{C}$ increases for the light intensities that are used here, bringing the actual temperature of the sample to 55–62 °C. This was confirmed by both thermocouples in solution as well as using infrared thermometers. However, the temperature changes can be dramatic if the SWCNTs are packed, as that can

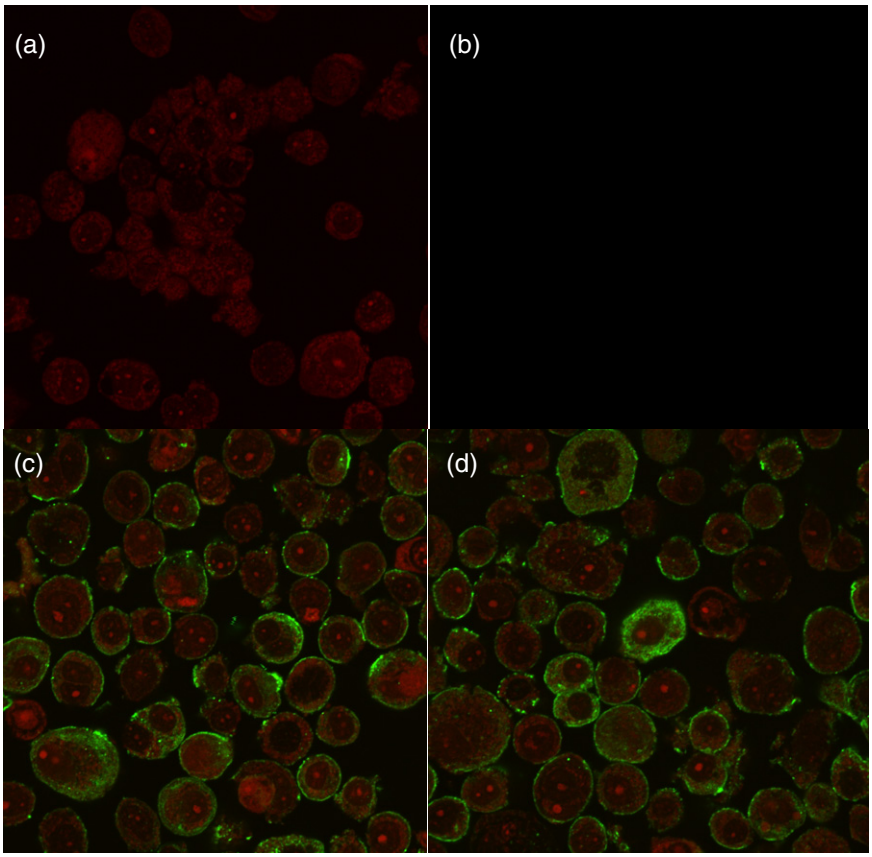

Figure 8. (a) Control group: SWCNT conjugated with Alexa Fluor 488 was completely terminated with PEG to prevent the binding with bio-molecules (no 1-pyrenebutanoyl succinimide was adsorbed on the side wall of the SWCNT). PEG/SWCNT conjugates were mixed with Alexa Fluor 555/anti-IGF1R conjugates and kept at room temperature for 30 min. MCF7 cancer cells were again incubated in such a solution for 30 min, after which the cells were triple rinsed and re-suspended to remove excessive fluorescent dyes. Under this configuration, almost no green signal could be detected on or inside the cells. (b) Second control experiment where only SWCNT and Alexa Fluor 488 was incubated with MCF7 cells for 30 min and confocal images showed diminished green colour. (c), (d) Repeat experiments of the internalization of SWCNT, scale bar: 10 μm.

trigger microscopic explosions in the room environment when reacting with oxygen from room air [6, 19]. The mechanism of light interaction on nanotubes is complex and poorly understood. Light causes interesting properties in nanotubes such as photo-conductivity due to exciton generation [24, 25], light induced elastic deformation of nanotubes, electrostatic charge separation [13] and explosions such as SWCNT nanobombs [6]. These effects are highly important for biomedical applications. For example, the explosions can not only be used for cancer therapeutics but also to generate acoustic waves at the nanoscale for the next generation of high efficiency ultrasound imaging applications. Stresses generated due to light on the surface of the SWCNT can also be used as a nanoscale delivery mechanism for proteins from the surface of the SWCNT into the cells.

The transduction of optical energy into the above mentioned energy domains can be high due to optical absorbance. The high optical absorbance of SWCNT is a result of electronic transitions from the first or second van Hove singularities [26]. While the electron–phonon coupling is negligibly small in arm-chair type metallic nanotubes, they have been shown to be considerably high in zig-zag semiconducting nanotubes. As a result the van Hove-like singularity in the density of states moves towards the top of the valance band enhancing the effective density of states near the Fermi energy [27]. This results in an increase in the electron–phonon interaction, thereby increasing the temperature of the nanotube. This also shows that semiconductive nanotubes are much better for applications in photo-dynamic therapy than metallic nanotubes. SWCNT are supra-molecular structures and the molecular vibrations can be intense on light absorption. For applications in selective cancer therapy, more than actual temperature increases, the thermal gradients are also important so that heat is only generated on the cancer cells while the surrounding normal cells are maintained at 37 °C. The thermal gradients in the nanoscale materials such as SWCNT can be extremely steep ($\sim 10^6$–10^9 °C m^{-1}). This means that while the nanotubes can heat up very locally to high temperatures, the steep thermal gradients localize the heating effects down from a couple of nanometres to micrometres, therefore resulting in a highly localized cell killing effect as seen in our experiments.

These results show that by targeting surface receptors and NIR dosing, one can achieve a high degree of efficacy in destruction of cancer cells. While past studies have shown the utility of nanotubes to transport DNA and act as NIR agents to kill cells, this is the first study to show the multi-

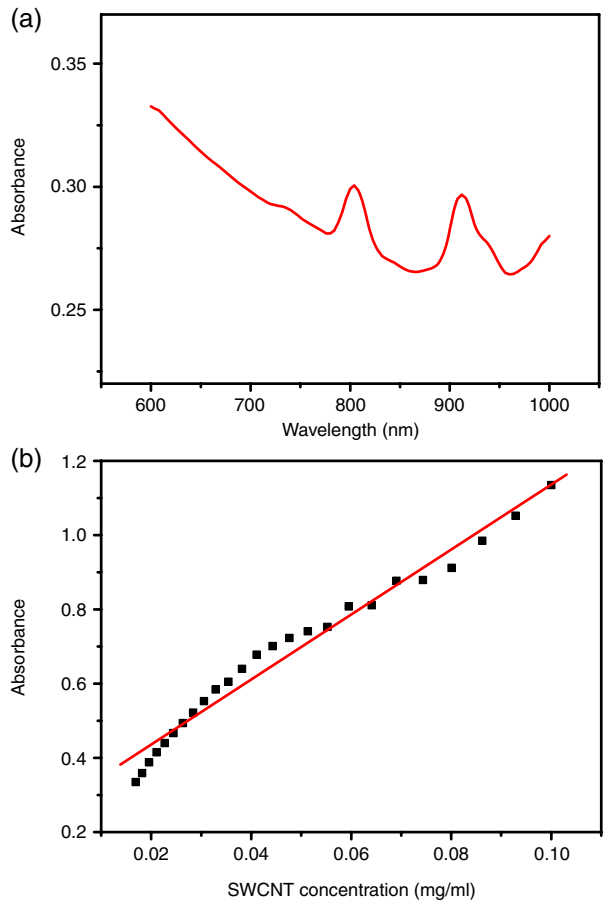

Figure 9. (a) Visible and near-infrared spectra for 0.03 mg ml^{-1} SWCNT functionalized by IGF1R antibody. Peaks at ∼808 and ∼910 nm indicate the high absorbance of the complex in the NIR region. (b) Absorbance of SWCNT of various concentrations in PBS solution. The solid line is the linear fit.

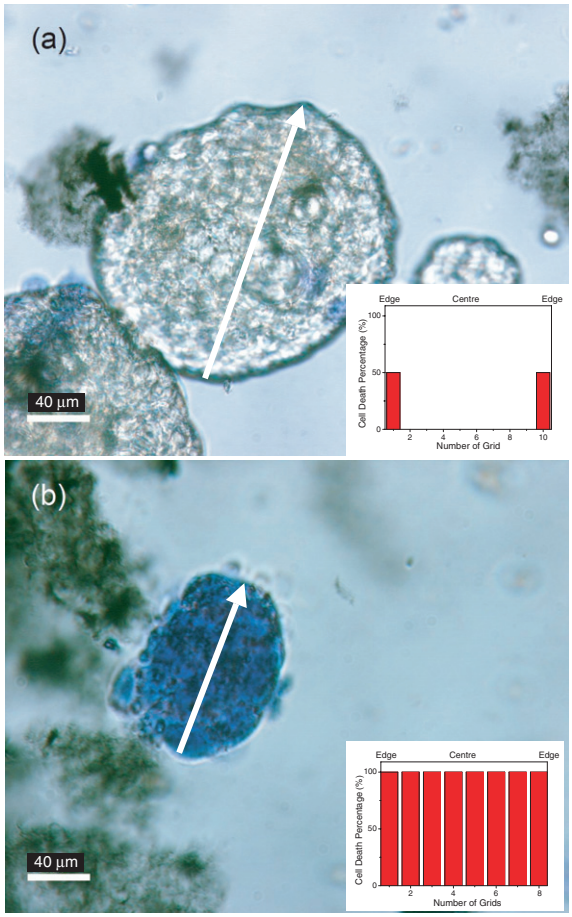

Figure 10. (a) MCF7 cancer cells treated without nanotube–non-specific antibody complexes survived the NIR dosing at 800 mW cm^{-2} for 3 min. Trypan blue was used to investigate membrane permeability and while the background looks blue, the cells appear white and reflective due to the impermeability of the Trypan blue. (b) MCF7 cancer cells treated with nanotube–anti-IGF1-HER2 antibody complex showing that all the cells died after NIR dosing at 800 mW cm^{-2} for 3 min. The cells appear blue in colour indicating cell death. Nanotubes start to precipitate as the samples started to dry around the cells. The diameters of both cell clusters (indicated by white arrows) have been divided into 20 μm grids. The percentage of dead cells in each grid has been estimated and plotted in the insets.

component targeting of more than one surface receptor (IGF1R and HER2) using SWCNT and simultaneous photo-dynamic therapy due to their high optical absorbance. The energy used in the destruction of cancer cells can be estimated to be ∼200 nW per cell, too low to create any damage to the normal cells compared to past nanoparticle and nanotube based cell killing techniques [7, 11]. Past nanoparticle and nanotube based cell killing techniques have used 4–35 W cm^{-2} and 1.4–3 W cm^{-2} for 3–4 min producing temperature changes for cell killing. Our method uses half the laser power of past nanotube based cell killing techniques and a 5–44 times lower laser power compared to past nanoparticle technologies. High internalization of nanotubes into the cells therefore minimizes the amount of energy necessary for killing the cells.

Past nanotube studies have targeted the folate receptor which is overexpressed in folate positive cancer cells and nanoshells have targeted more breast cancer relevant HER2 surface receptors. We have taken this a step further by showing multi-component targeting integrated with photo-dynamic therapy. By using nanostructures as biological transport carriers to target more than one surface marker, one can increase the efficiency of therapeutics that can lead to higher selectivity. Multi-component targeting and internalization followed by photo-thermal cell killing can therefore improve the efficiency and selectivity of therapeutics. A wide variety of proteins and molecules capable of overcoming biophysical barriers can be functionalized on the surface of the nanotubes as transport carriers. Nanotubes act as nanoscale thermal sources for material processing at the nanoscale and also for changing the kinetics of reactions of proteins inside cells. These could become the building blocks of non-invasive future cell repair machines using nanotechnology and optics.

5. Conclusion

In this paper, we have demonstrated multi-component molecular targeting of surface receptors (IGF1R and HER2) and NIR dosing of cancer cells using SWCNT. While previous studies have shown the transport of DNA into cells using nanotubes, in this study we show multi-component molecular targeting of both IGF1R and HER2 surface markers

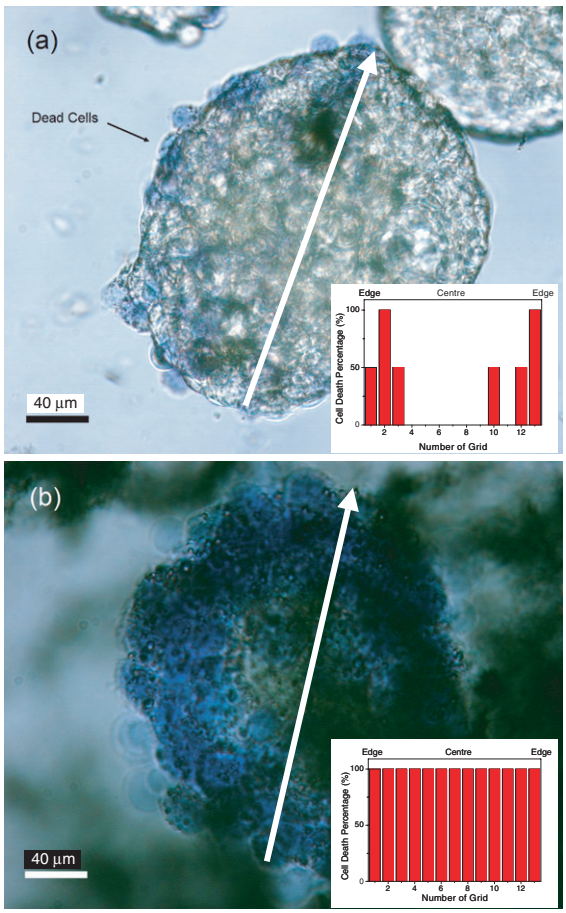

Figure 11. (a) MCF7 cancer cells treated with non-specific IgG antibody–nanotube complex did suffer from some damage as the collateral cells died after the laser excitation. Such a limited killing effect was due to the unselective binding effect of conjugates. (b) Repeated nanotube–anti-IGF1-HER2 antibody–nanotube complex MCF7 cancer cell destruction. Results show high repeatability of this experiment.

in cancer cells using SWCNT. IGF1 and HER2 specific antibodies conjugated to the SWCNT were used to target their corresponding receptors in cells and internalize the SWCNT. The cells were then dosed with NIR 808 nm photons at ∼800 mW cm^{-2} for 3 min. Cells that were treated with non-specific antibody–SWCNT hybrids survived the NIR dosing compared to the dead cells with anti-IGF1–anti-Her2–SWCNT hybrids. The amount of energy consumed for cell killing was estimated to be as small as ∼200 nW per cell, which is an order of magnitude less than competing techniques. Quantitative estimates showed that cells incubated with SWCNT–anti-Her–anti-IGF1R antibodies were completely destroyed while 80% of the non-specific-antibody–SWCNT hybrid treated cells were still alive. These results indicate that SWCNT could be used as biological transport agents and the high optical absorbance in the NIR is capable of killing cancer cells with minimum collateral damage. Further, western blots indicated the relevance of targeting IGF1R in MCF7 cancer cells and HER2 in BT474 breast cancer cells. This study is another clear example of the molecular targeting and cell killing approach that can improve therapeutic efficacies. These tools can also be applied to traditional surgical oncology to improve the probability of killing cancer cells whose microscopic foci are often left behind in surgery.

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